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How much coal?

There is not the slightest doubt that an immense amount of coal resides under the British Isles. Onshore there are at present many healthy coalfields, and new ones such as Selby, Belvoir and Coventry-Kenilworth could make substantial additions; further down the line the Oxfordshire field could possibly outshine anything that has gone before. Offshore, there is ample evidence of rich fields at great depth below the North Sea. Why then should anyone disagree with the claims of the National Coal Board (NCB), repeated recently in large advertisements, that 'we have proven coal resources to last for at least another three hundred years'? Is it not just an academic quibble to ask for more scrupulous definitions of the term 'proven resources'?

A wide range of geological opinion thinks not. The Flowers' Commission on Energy and the Environment has recently been turning its attention to coal, and has received some tart comments about NCB publicity — this is a dispute which has simmered for many years. Terminology in the assessment of minerals is, if you will pardon the phrase, a minefield. But common practice is to distinguish, first, between reserves and resources. The term 'resource' covers all material about which something is known, although information may be exceedingly thin either about the extent of the resource or about ways in which it could be exploited. And, not surprisingly, a 'resource' includes a large amount of material which will on further research, turn out to be unexploitable because of, for instance, environmental or social unacceptability. So one thing the NCB does not have is a 'proven resource' — a contradiction in terms.

What the board is actually talking about if it uses the word 'proven' is 'reserves': materials that have been mapped out sufficiently well that they can be the subject of mining by known methods. The most important of the several definitions of reserve is the 'workable' or 'operating' reserve — the amount of coal that can be mined economically according to present-day detailed assessment. This figure, like all reserves, may fall if the price of coal were to drop, and may fall just as easily as rise if new equipment were installed that required thicker seams or was sensitive to faulting. In 1973 the NCB's assessment

of current workable reserves amounted to 3.9 billion tonnes according to the board's then Chief Geologist (G. Armstrong, *Phil. Trans. roy. Soc.*, **276**, 439-452 (1974)). This is enough for around 30 years at present rates of consumption. Since then a vigorous programme of exploration and assessment has inched the workable reserves up to 6 billion tonnes.

How, then, does the NCB get its figure of 300 years — equivalent to a workable reserve of 45 billion tonnes? Geologists outside the NCB claim that the board tends to be a law unto itself and there is a severe shortage of coal geologists outside the board's embrace able to provide an independent estimate. But one thing does seem to be clear — that the 45 billion tonnes is bound to contain a vast amount of coal that is no more than rough guesswork at present. It seems that the figure arises not from the building up of detailed estimates from individual fields but from an estimate of what could be the total coal (shallower than 4,000 feet and in seams greater than 2 feet thick) under Britain; the total is then cut down to reflect the fraction of this coal which, on past experience, has actually been brought to the surface. The picture of a coal industry progressively and steadily working its way through the production of 45 billion tonnes over the next 300 years is thus, at best, no more than a giant extrapolation; at worst it could be totally deceptive.

But does it matter? Shouldn't the NCB be allowed a certain amount of exuberance concerning its future? In private, yes; there is little harm in a bit of optimism about an energy source that Britain has somewhat neglected in the recent past. But continued public statements on the subject could have a very harmful effect, as investment in energy technology and the development of other types of energy sources is bound to be distorted once the NCB's point of view gains general currency, particularly amongst politicians.

Most prudent energy industries like to look ahead at least fifteen years, and whilst NCB is talking about figures such as 6 billion tonnes it is being equally prudent. In making guesses at much larger figures and calling them 'proven resources' it is surely taking quite unnecessary liberties. □



Developing world 'a permanent desert for research'

Six countries account for 85% of the world's total spending on research and development. This monopoly is a major feature of global inequality, writes **Anil Agarwal**

MOST countries in the world today form a permanent research desert, the remaining few forming a small number of R&D oases. This is the conclusion of Dr Jan Annerstedt of Roskilde University in Denmark who has conducted a detailed survey of R&D activities across the world. Six nations (USA, Soviet Union, Japan, West Germany, France and the United Kingdom) employ nearly 70% of the world's R&D manpower and spend nearly 85% of R&D funds. The USA and Soviet Union account for more than half of the world's total R&D expenditure. Together, these six countries spend a quarter of every R&D dollar in the world for military purposes.

This "concentration of research and experimental development to a small number of highly industrialised countries is one of the major features of global inequality", says Dr Annerstedt in his paper on the present global distribution of R&D resources, recently released by the Vienna Institute for Development. Developing countries account for nearly 70% of the world's population but they contribute less than 3% of the world's R&D expenditures, and about 13% of the world's R&D scientists and engineers.

As far as the developing countries are concerned, this distribution of world R&D activity has remained almost unchanged. Based on a sample of countries for which R&D statistics existed in 1963 — 1964 (excluding most socialist and developing countries), the United Nations had estimated that developing countries accounted for only 2% of the world's R&D expenditure. The US was responsible for 70% and other market economies for the remaining 28%. Using this same sample of countries, Dr Annerstedt finds that their research expenditures have increased from less than \$30 billion in 1963-64 to over \$60 billion in 1973. But the share of developing countries has only increased from 2% to 2.8%. The share of the US has dropped from 70% to 50.7%, while that of the other developed market economies has increased dramatically from 28% to 46.5%.

Annerstedt's own survey of world R&D expenditures in 1973, however, also covers socialist countries in Europe and Asia and several more developing countries. He estimates that the world spent a total of about \$96.4 billion on R&D in 1973. Developing countries accounted for 2.9% of this sum. The rest was spent by rich countries, primarily in Europe and North America.

Annerstedt's data shows that within the Third World, South and Central American countries were spending most money on R&D in comparison to their populations —

about 33% of the Third World's R&D expenditure on 11% of its population. Asian countries (excluding Japan, Israel and Turkey) spent over 50% of Third World R&D expenditure but they contain over 75% of the Third World's population. African countries contain 14% of the population and spend about 10% of the R&D resources.

The distribution of R&D manpower is more in favour of the Third World than the distribution of R&D expenditures. Annerstedt's study shows that developing countries had more than 12% of the world's scientists and engineers engaged in R&D, or 290,000 of the world's 2.3 million researchers. Three quarters of the Third World's R&D workers were active in Asia, about 10% in Africa (excluding South Africa) and the rest in Latin American countries. The latter, however, had the highest number of researchers per million economically active persons (EAP) that is, 460 R&D workers per million EAP.

But if all R&D personnel are included (that is, technicians and other supporting

staff), then the Third World fares even better. The Third World, and in particular Asian organisations, have a higher number of technicians and supporting staff per researcher than the developed countries have.

Annerstedt believes that the distribution of overall scientific and technical manpower may be changing fast in the favour of the Third World. Educational expansion is faster in the developing than in the developed countries. In 1973, there were 4.6 million students graduating from universities and other institutions of higher learning all over the world. 23% of the third level graduates were from the developing countries (not considering those who were foreign students in developed countries and excluding China because of lack of reliable statistics). Among the twenty countries in the world with the highest total number of university graduates were seven developing countries: India, Brazil, the Philippines, Bangladesh, Democratic Republic of Korea, Egypt and Pakistan. The Philippines, Lebanon, Argentina, Venezuela and Chile had 54,500 to 48,050 third level students per million economically active persons. When compared in this way, they were only

Table 1: Distribution of world R&D expenditures among major regions and by average share of gross national product and per economic active person, 1973

	R&D expenditures: in mn US dollars	— in % of world total	per EAP in US dollars	in % of GNP at market prices
WORLD Total	96,418	100.0	66.4	1.97
DEVELOPING COUNTRIES	2,770	2.9	3.0	0.35
Africa (excl. South Africa)	298	0.31	2.8	0.34
South and Middle America	902	0.94	9.0	0.37
Asia (excl. Japan)	1,571	1.63	2.1	0.34
DEVELOPED COUNTRIES	93,648	97.1	182.1	2.29
Eastern Europe (incl. USSR)	29,509	30.6	160.0	3.82
Western Europe (incl. Israel and Turkey)	21,418	22.2	135.1	1.55
North America	33,716	35.0	331.1	2.35
Other (incl. Japan, Australia)	9,005	9.3	129.8	1.76

Source: Jan Annerstedt, *On the present global distribution of R&D Resources*, Vienna Institute for Development, Occasional Paper No. 79/1

Table 2: Distribution of researchers (R&D scientists and engineers) among major regions and per million economic active population, 1973

	Researchers total (000)	(R&D scientists and engineers): in % of World total	per mn EAP
WORLD Total	2,279	100.0	1,570
DEVELOPING COUNTRIES	288	12.6	307
Africa (excl. South Africa)	28	1.2	271
South and Middle America	46	2.0	461
Asia (excl. Japan)	214	9.4	292
DEVELOPED COUNTRIES	1,990	87.4	3,871
Eastern Europe (incl USSR)	730	32.0	3,958
Western Europe (incl Israel & Turkey)	387	17.0	2,441
North America	548	24.1	5,386
Other (incl Japan, Australia)	325	14.3	4,687

behind the US, Canada, Israel and New Zealand.

In terms of sheer numbers, India claims to possess the world's third largest scientific and technical manpower, only behind the US and USSR. India had at the end of 1977, 2.3 million people with qualifications in science, engineering, medicine and agriculture. In April 1976, 54,000 professional scientists were engaged in R&D work supported by some 40,000 technicians and another 50,000 people engaged in administrative and non-technical jobs.

China too has a vast stock of scientific and technical manpower. Aqueil Ahmed of the Administrative Staff College of India believes that India and China should be alternatively either third or fourth in international comparisons of scientific manpower. The eight-year science and technology plan (1978-85) recently announced by the Chinese government aims to increase the number of professional R&D scientists to 800,000 (compared to the total Third World complement of 288,000 in 1973).

But the fact that developing countries spend so little (2.9%) on R&D even though they have a much larger proportion of scientists (12.6%), means that each R&D worker in the developing countries gets few resources to work with. Many scientists have, in fact, emigrated from the Third World to the developed countries complaining that they do not enjoy adequate support. □

Military research the big spender

MORE than a third of the global research and development budget is spent on military and space research, and less than a tenth on health and agricultural research, according to a report published this week by the Worldwatch Institute in Washington. In addition, over 95% of R&D funds are spent in the industrialised countries; yet these contain less than 20% of the global population.

The report, written by Colin Norman, a senior research fellow at the institute, covers ground similar to the Annerstedt report (see left). Norman says that imbalance in research priorities — both between subject areas, and in meeting the needs of the rich rather than the poor — urgently need to be reordered. It points out, for example, that the US Government is expected to spend about \$670 million in 1979 on research and development aimed at improving the productivity of American agriculture; and that this sum far exceeds the agricultural R&D expenditures of all the developing countries put together.

"The worldwide distribution of R&D capacity closely matches the global distribution of economic power," says the report. The disparity applied both to financial and manpower resources. It had been calculated, for example, that during the early 1970s developing countries had about 300 scientists working on R&D for

every million workers, compared to the developed countries, which had 4,000 scientists for every million workers.

The report says that the world's research and development priorities need to be reordered by, for example, channelling more money into neglected research areas, new research organisation and Third World laboratories, as well as encouraging the increasing expenditure by industrialised countries on research appropriate to developing country needs, and stimulating co-operative research efforts between the developing countries themselves.

However, the report warns that such efforts will not, by themselves, be sufficient to solve the world's major problems. "Many tasks are too urgent to wait for R&D to provide solutions and many cannot be solved by science and technology alone. Indeed when new knowledge is used to bolster and extend the power of governments, corporations and ruling elites, it can aggravate the social injustices that lie at the root of many of the world's most urgent problems," says the report. □

Knowledge and Power: The Global Research and Development Budget, by Colin Norman. Worldwatch Paper 31. Available from the Worldwatch Institute, 1776 Massachusetts Avenue NW, Washington DC 20036.

New US group calls for gene resource conservation programme

A DIVERSE group of scientists, concerned over the lack of support for efforts to protect genetic resources met recently in Berkeley, California, to form the National Gene Resource Program Co-ordinating Committee. The committee will initiate a train of events which, it hopes, will culminate in a national conference on gene resource conservation in 1981.

The committee's first action was to circulate a letter requesting other scientists and people concerned with bioresources to bring the problem to the attention of the federal government.

In an interview with *Nature*, the co-ordinator of the committee, geneticist David Kafton, elaborated on the goals of the group. "What we envision is making gene resources a high-priority national issue," he says. The task of the new committee will be to "provide a vehicle for effective action — not just talk."

Critics of the so-called Green Revolution have long pointed out the double-edged danger of over-reliance on just a few strains of essential crops: the chance that disease could wipe out whole harvests very suddenly and that further breeding potential could be lost forever as wild strains disappear. The devastation left by flare-ups of wheat rust, southern corn leaf blight and Dutch elm disease in the United

States in recent years has illustrated this point.

Some action has been taken. The National Seed Storage Laboratory in Fort Collins, Colorado, has pioneered practical genetic preservation by storing and protecting seeds of more than 100,000 kinds of food and fibre plants. Bernard Finkle, one of the founding members of the new committee, and his colleagues at the Western Regional Research Laboratory of the US Department of Agriculture in Albany, California, have been experimenting with methods of storing in liquid nitrogen the individual cells of plants usually propagated by cuttings rather than seeds.

Such efforts, however, are too poorly funded to assure the preservation of genetic variability for even essential crops, Kafton says. He cites a memorandum sent last March to Secretary of Agriculture, Robert Bergland, by the National Plant Genetic Resources Board — an advisory body to USDA — outlining the need for greater effort in gene conservation. The National Academy of Sciences has also warned that the "genetic diversity for many species is severely threatened" and has recommended the setting up of a new agency for preserving germ plasmas.

Meanwhile, an estimated 200 plant species disappear every year, particularly in

areas of developing countries where land is cleared to raise fast-growing new hybrids only. Current levels of sample collection preserve only a small fraction of the existing natural diversity of crops and forests, Kafton and his colleagues insist, and too little research is directed towards finding new methods of conservation.

The letter they are now circulating — really a petition for action — calls for "the establishment of an effective and comprehensive conservation program to ensure the protection of this nation's irreplaceable and invaluable gene resources." Specifically they call for a national conference "whose primary task will be to initiate and design such a comprehensive program."

Technical support for the committee, and other action groups like it, is being supplied by a small, new, non-profit firm called Inquiring Systems Inc., of Berkeley. Loren Cole, an ecosystemologist, is executive director of the company, and David Kafton is one of the staff scientists. Kafton says that the new company is designed to work with groups like the new gene resource committee to stimulate action on social issues through such activities as organizing meetings, training personnel and publishing information.

John Douglas

UN studies new science and technology fund

Keen to avoid an impasse at the United Nations Conference on Science and Technology for Development when it meets in Vienna next month, UN officials are studying the idea of setting up a new mechanism to support activities meeting the goals established by the conference in a way that is acceptable to both developed and developing nations.

The Group of 77 which is currently negotiating such issues on behalf of over 120 developing countries, has already proposed that an international 'financing system' for science and technology for development be established by the UN, largely financed by the developed nations and aimed to grow to \$2,000 million by 1985, and \$4,000 million by 1990.

Many developed countries, however, have pointed out the political difficulties that they would face in committing themselves to such a fund, both in terms of the size of the contributions involved, and the form in which it is proposed that they be made — namely as 'automatic' contributions assessed, for example, as a percentage of the balance of trade in manufactured goods with the developing countries.

What is now being sought is a formula that might eventually lead to a transfer of funds for scientific and technological activities of the same order of magnitude as the Group of 77 have proposed, but in a form which would be politically acceptable to the countries expected to be the major donors.

A possible way in which this could be done, according to one line of argument now being actively pursued in the UN and elsewhere, is through an initially relatively modest financial allocation — perhaps in the order of \$250 million to \$300 million — which could both provide some immediate additional support for science and technology activities and establish the basis for exploring the desirability and feasibility of scaling up to the higher budget levels.

The need to increase the finance for science and technology in development has been emphasized in a paper presented to the UNCSTD secretariat by the United Nations Development Programme. One of the suggestions made in this paper is that the conference should examine and, if appropriate, establish "international arrangements to generate and channel additional funds to developing countries . . . to stimulate research and development activities in priority areas."

The UNDP suggests, for example, setting up an international financing facility which, at the request of a particular country, would support selected research projects in key areas, as well as the development, field testing, pilot production and dissemination "which are so essential in the conversion of research into widespread practice"

Such a facility, it says, would aim to ensure that adequate resources were channelled into key areas, and would arrange to recover its costs in whole or in part in sectors such as industry, where appropriate. It would also attract funds from non official sources, combining an intergovernmental mechanism with a mixture of public and private funds through co-financing.

"If the facility were both effectively managed and secure, it could attract, through co-financing, substantially greater levels of funding than are likely to become available from official sources alone. It would thus provide an important new source of support for a variety of critical links in the development process in many developing countries", says the UNDP.

Addressing a meeting of the development assistance committee of the Organization for Economic Co-operation and Development in Paris two weeks ago, Mr Bradford Morse, UNDP administrator, stressed the importance to developed and developing nations alike of a successful outcome to the UNCSTD conference, for substantive economic and political reasons.

He stated that the UNDP was prepared to upgrade its own scientific and technological activities in line with the general goals agreed by the conference. And he also suggested a possible scenario for agreement between UNCSTD delegates that would include

- The immediate provision of additional funds to support scientific and technological activities in developing countries;
- A commitment by the developing countries to work out their most pressing needs in science and technology;
- A careful review of alternative financing mechanisms for meeting these needs, and how these could be built up; and
- Investigation of ways to ensure the adequate involvement of the scientific community in development efforts.

Speaking at a joint Senate and House hearing in Washington last week, Mrs Lucy Benson, US Undersecretary of State for Science and Technology, said that US negotiators at the UNCSTD preparatory committee meeting in New York last month had considered the Group of 77's proposal of a financing system was "somewhat unrealistic".

"We all feel that we will not be able to come up with what is considered an adequate sum of money" Mrs Benson told the congressional hearing. However, she said that members of the US UNCSTD delegation were waiting to hear more about the plans being discussed in the UN for a funding facility along the lines suggested by UNDP, and in particular were waiting for decisions to come out of the executive branch about the type of commitments

that the US would have to make in Vienna.

Ambassador Jean Wilkowskizn, US co-ordinator for UNCSTD, told the meeting that there was a suggestion within the UN that a new science and technology facility, if established, might "very much involve the Arab nations".

Officials in Washington last week confirmed that, in principle, those responsible for allocating aid funds within the US are sympathetic to some new type of facility being established for science and technology, particularly since it might parallel on an international basis the type of bilateral activities proposed for the Institute for Scientific and Technological Co-operation (whose fate, following its rejection by the Senate two weeks ago, still hangs in the balance).

The rationale for the ISTC has already been successfully argued for within the administration. On an international level, many may feel that a logical place for such a facility would be the UNDP. Much will depend, however on what type of financial commitment the administration feels that it is able to make in both the short and the long term.

Meanwhile, some speakers at the congressional hearing used the opportunity to express their frustration at the apparent lack of initiatives which the US seemed to be proposing to take at Vienna. "Our objectives for Vienna are fine, but the effort we proposed is scarcely commensurate, unless things change in the next few weeks", said William Carey, executive officer of the American Association for the Advancement of Science.

Mr Thomas Pickering of the State Department, however, told the hearing that the administration had been studying a wide range of possible initiatives, and was making a final selection of those it considered most feasible. Prime candidates are thought to be plans for resources monitoring satellite systems for developing countries (using technology developed by the National Aeronautics and Space Administration), a proposal to assist in setting up a global information network, and plans to link ISTC — if approved — to similar institutions in other developed countries.

Finally, asked whether the developed and developing nations were on the same wavelength as far as negotiations for UNCSTD were concerned, ambassador Wilkowski gave a frank appraisal of the situation. "It came as a shock to the National Academy of Sciences when it realised that this conference is not about science and technology but is political, because it deals with developing country concerns about inequality and dependence. Those are the catalytic forces which have to rise to this conference. It is the desire to overcome them which led to UNCSTD."

David Dickson



UK energy minister backs conservation and nuclear power

"We must have a balanced energy policy, drawing on every one of the resources available to us", David Howell, Britain's Secretary of State for Energy, told a gathering to celebrate 25 years of the UK Atomic Energy Authority last week.

Mr Howell (above) backed nuclear power — without it, he said, the UK "would not have a realistic policy for meeting this country's long-term energy requirements". But he also stressed the need to tap coal, and conservation, "where savings of 30% or more are available". Mr Howell announced that he was reviewing the UK conservation programme "to see what reinforcement may be necessary in this key part of our energy strategy" □

UK fellowship scheme approved — in principle

BRITAIN'S Science Research Council has given general approval to the establishment of a programme aimed at easing the critical staff stagnation problem which now affects the country's universities. However, its central council last week decided to call for more specific details before giving the final go-ahead to the scheme which would provide special release fellowships to help senior research workers vacate tenured posts that could then be filled by young scientists.

There are a number of drawbacks associated with the scheme but council members were particularly concerned that the programme, which could cost up to £450,000, could give universities an excuse to cut back on their own financing of science departments and transfer funds to other faculties. To prevent this, more specific regulations are now being prepared and the plan will be discussed by the SRC again in the autumn. □

China eases ideological grip

"CLIMATE" science in the last sixty years" is the title of an article appearing in a recent issue of *Hong qi (Red Flag)* written by Chou Pei-yuan — who is in charge of the Chinese Scientific and Technological Association.

Chou expresses his opinion on the further progress of Chinese science, and refers to the role of marxism. While no substitute for science proper, materialism and the dialectics of nature nevertheless constitute a powerful methodological tool in scientific research. He quotes the 'straton' model of hadrons in particle physics and the chemical synthesis of biologically active insulin as examples of advances made by Chinese scientists under the guidance of marxian ontology.

On the other hand, real scientific discoveries may also be made by experimentalists who are not consciously materialists and dialecticians. In the development of any discipline, the general policy should be "let a hundred schools contend", he says. Academic freedom includes the understanding that no scientific model or theory will be rejected, or supported, purely on the basis of politico-philosophical arguments. Lysenko-type mistakes, such as the uncritical refutation in the late 1950s of western psychology, must be avoided.

Chou also reviews the slow and uncertain development of science in China before 1949, when research was hampered by the lack of experimental facilities, and when significant original work was confirmed to geology, biology, palaeontology, archaeology and

mathematics. He describes how Chinese science developed in the quarter century after 1949 with the socialist transformation of scientific and educational institutions. However, in the last few years, research of a fundamental nature has been largely neglected, and, he says this needs to be remedied quickly.

Chou's final point about the position of scientists is perhaps the most problematical. He mentions the phenomenon of being "hot at both ends but cold in the middle": that the enthusiasm for scientific modernisation among the top leadership and the rank-and-file scientists does not find its way to the mid-level cadres in some parts of the country. He argues that the majority of intellectuals in China are now part of the working class, and that more say in the administration and funding of science should be given to the scientists themselves. They should have the right working conditions so they can concentrate on research. Other problems which need attending to are the rational allocation of research duties to specialists and the implementation of arrangements to reduce the amount of time they spend on household labour. **T.B. Tang**

●China is to spend £3,500 million on cultural, educational, scientific research and health undertakings this year. Nearly half of it will be devoted to the running of institutes under the Academia Sinica and to maintaining scientific research various other departments, according to the latest state budget announcement. □

Yugoslavia: no more oil burning

YUGOSLAVIA'S Federal Chamber last week passed an emergency law, banning for the time being any further investment in new power stations and heating plants based on liquid fuel. This move follows a report from the Federal Executive Council which noted that, as a result of increased OPEC prices, Yugoslavia's oil import bill for 1979 will be 37,650 dinars, some 72% higher than the original estimate. Furthermore, increased domestic consumption is unable to keep up with existing supplies, so that last month, for example, certain Yugoslav airports were closed for several days, due to a total lack of aviation fuel.

The emergency bill, however, did not have an easy passage. Originally scheduled for 27 June, it was held up for three weeks by the delegates from Vojevodina province, who demanded a special procedure and moratorium, claiming that the law would endanger the vital interests of the province, and that the situation did not justify a state of emergency. Vojevodina has small but promising oil resources of its own, and recent prospecting operations by the Naftagas

enterprise suggest that in the future, Vojevodina may be able to supply up to 5% of the crude oil required by the Belgrade, Novi Sad and Pancevo refineries.

In spite of these discoveries, Yugoslavia's energy planners are rapidly rethinking their strategies to include new developments of the abundant lignite reserves and a re-emphasis on the country's coal mines, many of which have been closed in recent years. Nuclear power, too, receives a new emphasis, and it is hoped to commission one new nuclear plant a year, from 1990 onwards. However, additional funding for the first such plant, at Krsko, is still on the "pending" list of investment, while the proposed site of the second such station on Vir island has had to be abandoned, following protests by local residents and tourist enterprises. As an extraordinary measure, the station will now be built at Prevlaka, a village on the Sava near Zagreb, although Rade Pavlovic, head of the Croatia Electricity Board, stresses that all such stations in the future will have to be built on the coast.

Vera Rich

Water quality threatens Polish agriculture

Following an unusually severe winter and dry spring, Poland faces a short-fall in the harvest, a situation in which Party Leader Edward Gierek, meeting agricultural 'activists' earlier this month, could only advise them to "take a close look at every field and harvest as much grain as possible. We must not run round looking for possible places where grain can be bought. Firstly, the money is not there, and secondly, we cannot buy grain anywhere".

Although attributable in the short term to exceptional climatic conditions, this short-fall is a symptom of one of the major problems bedevilling the Polish economy, a chronic lack of water. Not only do many parts of the country have an overall deficit of water, even where there is abundant rainfall, there is insufficient reservoir capacity to store it. In all, Poland's existing 140 reservoirs can hold only 2,800 million m³ water, some 5% of the mean annual flow of the rivers (for comparison, Bulgaria stores 15%, Czechoslovakia 12% and the Soviet Union more than 14%).

Although the new Vistula Programme, formally inaugurated on 18 June 1978, is designed to remedy this situation to give a storage capacity for the Vistula of approximately 6,000 million m³, in addition to modernizing the river's transport facilities and building hydroelectric stations. However, provision of water is not sufficient. The Vistula is now so polluted that for much of its length, the water is not even up to the 'second class' of cleanness required for agriculture. A spokesman for the Programme, told *Nature* that "the main purpose of the Programme is to provide the nation with

pure water. All the other aspects, transport, hydroelectricity etc, could be replaced by some other means. But without pure water there is nothing we can do."

Like all major technological undertakings in Poland, the Vistula Programme draws heavily on scientific potential throughout the country. Virtually all major Universities and Polytechnics are involved, especially Warsaw, Krakow and Wroclaw. Coordination is the responsibility of the Academy of Sciences.

Although work on the Vistula Programme is not due to start until 1981, the extreme urgency of the situation has led those involved to anticipate the official inauguration. A few administrators have already moved into the half-built office block in the Warsaw suburbs assigned to the Programme, while on the river itself preliminary dredging operations are already in operation. Yet even at this early stage, the engineering operations seem likely to be held up by environmental problems.

Plans for the river include floodwalls which will enclose the present channel and the water-meadows susceptible to flooding; dams will then be built between these floodwalls to create reservoirs with a minimum depth of 2m. The Institute of Ecology of the Academy of Sciences has decided, however, that no dams should be built until the water in that area is up to second class standard, a demand of unparalleled severity.

Cleaning up the Vistula to an extent where largescale engineering operations can commence will involve a considerable number of interests. In addition to the

routine problems of industrial outfall and domestic sewage, there is heavy mineral pollution from the mining and industrial belt of Silesia, to say nothing of the Nowa Huta Steelworks near Krakow, where the ecological price of the annual steel output of 7 million tonnes is an emission of 100,000 tonnes of dust and some 70,000 tonnes of SO₂.

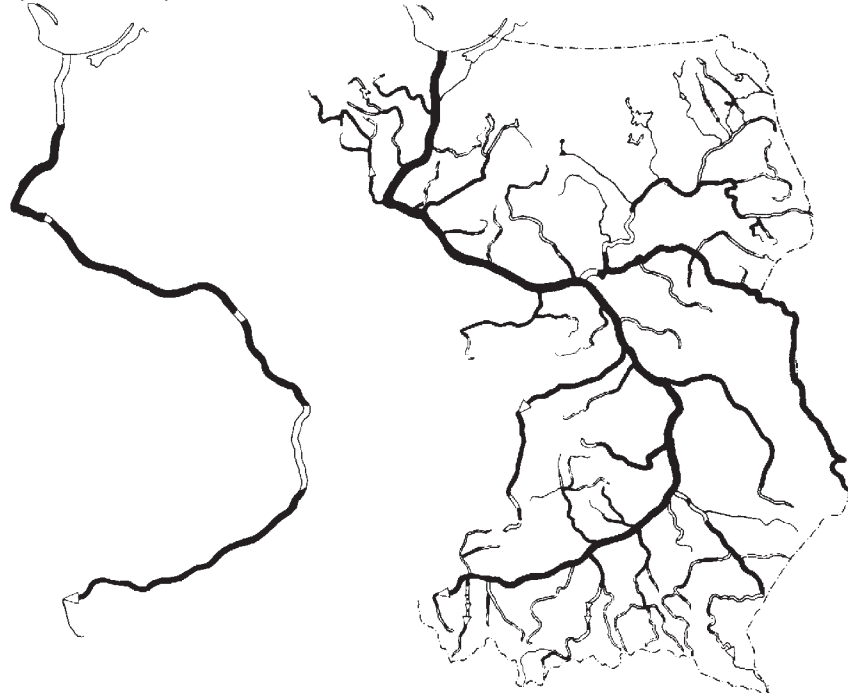
Many ecologists concerned with the Vistula Programme see this indirect pollution *via* soil and air as a greater problem than that of wastes discharged into the water, which can, at least in principle, be dealt with by improved sewage, recycling, oil-trap and purification systems. The Academy's Environmental Engineering Institute (situated appropriately, at Zabrze in Silesia), is working on a new electric filter, now at the pilot model stage. Filters and precipitators have in fact installed on all new furnaces at Nowa Huta since the mid-1960s; of them, Professor Roman Andrzejewski, of the Environmental Engineering Institutes Krakow office simply observed "The filters would be fine if they had an efficiency of 90%". What is really needed, he added is "a technology without dust and without gas!"

Until that utopian situation is achieved, the Institute of Ecology is trying to work out some ecological solution to the pollution problem. In Silesia, valuable data have already been collected on the uptake of minerals. Although it is far too early to propose a general strategy, some partial solutions have been suggested. Zinc pollution, for example, can be remedied by planting birch trees, which can apparently accumulate zinc without harm. Similar methods, say the researchers, can also be applied to agricultural run-offs.

To avoid eutrophication of the future reservoirs, they propose filter zones of live ecosystems on either side of the river. Alder woods are particularly valuable, as too is grass-land, which can profitably be used for grazing. Preliminary studies on the disposal of agricultural chemicals by such filters is going forward in the Mazurian lake district. Not only the absorption by flora is being investigated, but also the uptake and "export" of the chemicals *via* the small fauna.

But, faced with a pressing lack of water, as highlighted by the current harvest estimates, will the planners be prepared to wait? It is not, of course, the specific task of the Institute of Ecology to hold them off — but the consensus of opinion seemed optimistic. "We asked the engineers about this last year", said one ecologist, and they said that they would never put a dam where it would make the water quite dead!" "We fell sure that the planners will do what we suggest," said another, "they are actually trying to improve their contacts with us".

Vera Rich



State of the Vistula in 1967 and 1976: black areas are water unfit even for agriculture (data from the Instytut Kształtowania Środowiska, Wrocław)

news in brief

Army worms on the march: Reports from a number of sources indicate that army worms, second only to locusts as a threat to agriculture over much of Africa, are occurring in very large numbers this year in West and East Africa and in the southern Arabian peninsula. Army worms (pictured right), larvae of the Lepidoptera genus *Spodoptera*, attack food, especially cereals, fodder as well as cash crops such as cotton. But in East Africa the most serious damage may be to grazing. In earlier outbreaks, the pest have tended to move northwards in a series of waves and may cause a cattle famine anywhere from Transvaal to southern Ethiopia.

The current situation adds urgency to research on the possibility of using the *S. exempta* virus as a pesticide. This research was held up last year because no one could locate any army worm outbreaks. Use of *S. Exempta* is the second stage of the virus-and-pheromone concept, the first stage of which, directed against the Mediterranean army worm, (*Nature*, 7 December 1978) has now reached the stage of extended field trials on 5,000 acres in the Egyptian Fayoum — from Peter Collins in Geneva.

Soviet call for co-operation on global problems: Academician Dzherman Gvishiani, deputy head of the Soviet Union's State Committee for Science and Technology, has called for a "concerted approach" to the solution in Europe, and ultimately the world, of such major problems as environment, population, water resources, health care, and desertification. Writing in *Pravda*, Gvishiani stressed that such problems can no longer be solved by the efforts of one country alone. In this he differs markedly from Academician Anatolii Aleksandrov, chairman of the Soviet Academy of Sciences, who averred last April that "there is no scientific-technical problem that would not be within our power." Gvishiani, urges the setting up of all-European or inter-state forums to help turn the Helsinki accords into specific action for environmental protection, the development of transport, and energy.

Fire at Windscale causes radiation exposure: Eight workers suffered radiation exposure when a fire broke out on 17 July at a decanning cave at British Nuclear Fuel's Windscale reprocessing plant. The most serious case was 0.3 rem exposure, the equivalent of 10 chest X-rays. This is below the unit of 3 rem in any 13 weeks set for radiation workers by the International Committee for Radiation Protection. The fire occurred when a piece of irradiated fuel rod became jammed in a fuel assembly. Dismounting machinery failed to remove the jammed rod and the fire started, presumably due to friction between the assembly cover and the uranium rods. The fire was extinguished after 45 minutes by flooding the area with water manually through access hole in the top of the cave. Numerous questions remain to be answered about the incident, including the reason for the escape of material and the failure of safety provisions to prevent the event. Community representatives on a BNFL community liaison committee have called for a full investigation "on behalf of the local people and not as a public relations exercise for BNFL".

Czech scientists await trial: Five scientists — mathematician Vaclav Benda, psychologists Jiri Nemec and Dana Nemcova, and (engineers) Ladislav Lis and Petr Uhl — are among ten Czech human rights activists now awaiting trial for "subversion on a large scale". In plain terms, their offence is their membership of VONS, the "Committee for the Defence of the Unjustly Prosecuted", a subgroup of the Charter 77 movement. Since its

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inauguration, the committee has issued 113 statements on alleged breaches of justice and legality. The ten detainees were arrested on 29 May, and their trial is expected in the next few days. If convicted, they face prison sentences of up to 10 years.

No future for nuclear power plants in Iran: "Only a miracle can rescue both nuclear power stations" an official of the West German engineering company Kraftwerkunion GmbH said last week. He told *Nature* that work permits for the last 50 members of Kraftwerkunion staff will expire by the end of this month. Earlier in the year, there were still hopes that the 6.9bn works would be finished by 1981 the contract was 80% completed. But influential officials are taking a strong line against any nuclear power stations in Iran, and Kraftwerkunion now sees little hope of a resumption of work. The prospect of the plant's cooling towers ending up as grain silos now appears far more likely than before. — Klaus Höpfner.

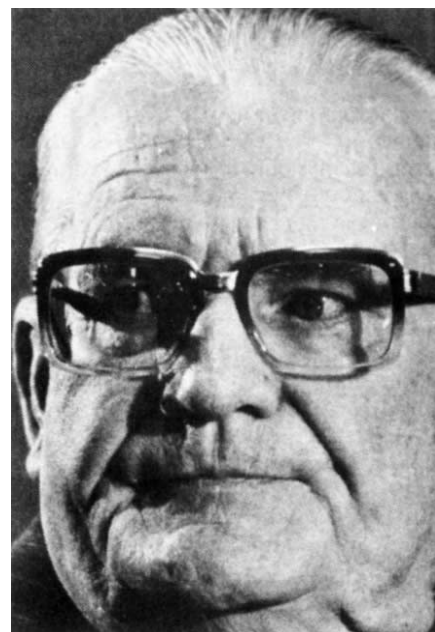
ILL ready for its "deuxième souffle": The renewal programme for one of the world's leading neutron research facilities, the Institut Laue-Langevin in Grenoble, is close to final approval. Funds for ILL's "deuxième souffle" — amounting to 104 million French francs to update instruments and techniques — were approved by the member countries, Britain, France and Germany on 25 May. But because of prior French budget commitments, the detailed spending for the five year programme has been postponed by the French until October. ILL Director John White calls the 25 May approval "extremely good news" and is optimistic that the French difficulty will be resolved.

Jupiter space shot survives budget threat: Project Galileo — NASA's plan to send a joint probe and orbiter mission to Jupiter in 1982 — has survived a threat from a key Congressional subcommittee, some members of which had been threatening to cut off funds to help pay for overspending on the space shuttle. The threat to kill the project came primarily from Senator William Proxmire, chairman of the Senate appropriations Subcommittee responsible for the budgets of both NASA and the National Science Foundation. Senator Proxmire, no great friend of the basic science community, had also suggested that about \$20 million be taken out of the budget of the proposed space telescope. However, at a meeting last week the subcommittee refused to go along with either of these proposals, and agreed to recommend to the full appropriations committee that NASA be granted the full \$3,822 million in the President's budget request for the fiscal year 1980. Within this total, however, the subcommittee recommended that budget cuts totalling \$17 million be allocated to five new projects, including preliminary design studies for a national oceanographic satellite system (a proposed successor to SEASAT), and a multispectral resource satellite.

Islamic technical training centre opens in Bangladesh: An Islamic Centre for Technical and Vocational Training and Research (ICTVTR) began work at Dacca last month, with a board of directors including representatives of Saudi Arabia, Libya, Iran, Iraq, United Arab Emirates, Turkey, Indonesia, Senegal, Gambia, and Bangladesh. The Centre will be built on a 30 acre site provided by the government of Bangladesh at Tongi, 10 miles north of the capital. It will cost \$9.6m and is expected to be completed by April 1983. Saudi Arabia has already announced a grant of \$2m for the centre. ICTVTR has drawn up a programme for a ten month interim training course in machine shop technology for 50 instructors; and to meet the technical manpower needs of Islamic countries it will undertake training programmes in chemical and mechanical technology, design and drafting, computer programming and technical innovations in oil and gas. — from M. Kabir in Dacca.

Brazil: is it really safe for the scientists to return?

Brazil's stern leader, President Ernesto Geisel (right), has relaxed some of the restrictions on academic freedom that forced many scientists to leave the country. **Maurice Bazin** reports on the sceptical reaction among the exiles.



THE Brazilian government has recently proclaimed that it will create a gradual democracy and re-establish the right of *habeas corpus*. With this emerges the possibility that some exiled Brazilian scientists may return permanently. Although many exiled scientists have returned on visits in recent months, it is not clear whether the military government really wants them or if they are being tolerated only temporarily. These scientists had been engaged in creating respected research institutions in the early 1960s, but their work was nullified by arbitrary firings and forced retirements imposed by a government afraid of 'intellectual subversion'. As a result, many have been forced into exile, contributing involuntarily to the very 'brain drain' which their earlier efforts in Brazil aimed at eliminating.

A point of convergence for all scientists, both in exile and in Brazil, is the annual meeting of the SBPC, the Brazilian Society for the Progress of Science, now taking place in Fortaleza, a coastal city in the north-east of Brazil. The motto of this year's meeting "Sing there while I sing here" shows how prominent the theme of reunification has become within Brazilian scientific circles.

Meetings of the SBPC in past years have been marred by conflicts with the military authorities who wanted to restrict their impact. Two years ago the government forbade all scientists working in federal institutions or using government funds from participating. The meeting's venue should have been the Federal University in Fortaleza, but it was forced to move to a private institution, the Catholic University in São Paulo.

In a well-publicised 'Declaration of Scientists to the Brazilian People', approved by acclamation at the 1977 meeting, the SBPC asked that "scientists fired through dictatorial decrees be reinstated." The declaration also stated that the SBPC "repudiates all censorship of artistic, literary, journalistic and scientific activity. In particular, it repudiates the recent decision of the government to impose censorship upon foreign publications. We see this as a retrograde action, negating the universal character of knowledge and forcing the cultural isolation of Brazilian intellectuals." On its 30th birthday, last year, the SBPC ironically asserted: "the revolution of 1964 was a great stimulus to our economy; the firings and deportations

of 1968 and 1969 were also. Each exiled professor costs the country 50 thousand dollars and another 50 thousand to import another who is not *non grata*."

The fact that this year's meeting will finally take place in Fortaleza is a positive step in the process of liberalisation but how many exiled scientists will attend and how many would resettle in Brazil are very much open questions. According to Professor Rocha e Silva, a founder of the SBPC, "some scientists are now living much better in foreign countries. The problem is not of an amnesty, but to convince those who receive the amnesty to come back." For others, however, it is not a question of their standard of living, but doubt about the liberalisation process itself.

"It is ironical that I should return to my country on the occasion of the SBPC meeting; it was during the Society's meeting in 1964 that I was arrested," says Professor Luis Pereira da Silva who now directs the Department of Parasitology of the Institut Pasteur in Paris. "I am going only to renew contacts with the Brazilian scientific community and meet old friends again." In 1964 Pereira da Silva was Assistant Professor in the Medical School of the University of São Paulo; his work on Chagas disease and the chemotaxis of tripanosomes had already received international recognition. He and eight colleagues were fired, with no justification given, under the First Institutional Act, a dictatorial decree of the military regime. At the forthcoming SBPC meeting in Fortaleza he will chair a discussion session entitled 'Who should be responsible for the orientation of scientific research'.

How did Brazil suffer such a massive loss of scientists in the mid-1960s? "We were all free intellectuals, independent thinkers. We wanted to set up modern institutions on a standard academic basis", says Roberto Salmeron, now Directeur de Recherche in high energy physics at the Laboratoire de

l'Ecole Polytechnique in Paris. He was one of the group who conceived the idea of the University of Brasilia, and in 1965 he was coordinator of its Science Division. "We wanted a democratic way of running a university. We used the word 'coordinator' to avoid the authoritarian 'director'. The university was to be a true community and teachers and students were to reside on campus — a very new situation for Latin America."

While Salmeron was trying to build up the science departments, the Rector of the university was changed twice. "The authorities named persons who had the confidence of the military and who were totally subservient. One day the Rector told me that I could not hire a certain professor; he had been called in by unspecified superiors and warned that my candidate was an 'agitator'. Our lectures were taped secretly and we could be asked to explain what we had said in class. Even the names of the composers whose magic was to be played at free public concerts on Saturdays had to be cleared in advance; a conductor was called in for having scheduled a Prokofiev work.

"Although the government started dismissing professors during the very first month of the military regime (13 social scientists were dismissed in March 1964) we succeeded in building up a Division of Science with 150 personnel by 1965." The



All calm in Rio — or is it just a temporary lull?

process of confrontation escalated when a new Rector announced that he would dismiss six other professors. "In spite of our efforts to explain directly to the highest military authorities the value to our country of a modern university, and despite the fact that their sons and daughters were attending it, those in power remained completely insensitive." The result was a series of assemblies of teachers and students, with talks of strikes; finally the army took over and sealed off the campus.

"Nobody could enter the campus, and all our research animals and the flies of the geneticists were going to perish. I alone obtained an authorisation, which read: 'Professor Salmeron is authorised to enter the grounds of the University of Brasilia'. I have kept this piece of official democracy to this day." Finally, towards the end of 1965, as the Rector demanded that Salmeron countersign the firing of not six but 16 scientists, he resigned instead and made public 250 other individual letters of resignation that his colleagues from other departments had prepared. "For five months, we tried to find work as a group of physicists in other Brazilian universities, away from the centre of power that Brasilia represents. We did not succeed and I finally came to Europe, accepting an invitation of Victor Weisskopf, then General Director of CERN."

'I have no confidence in this political overture'

As to whether he will return to Brazil now, Professor Salmeron says "I do not understand what is going on in Brazil. I have no confidence in this political overture — people only do what they are allowed to do. There is some influence coming from the intellectuals, but in the case of the nuclear agreement with West Germany, the influence of the scientists was zero. They were told only after things happened. One should not have illusions. I can only imagine going back in the distant future, and it would depend on the existence of a real stable change in the Brazilian situation which would allow me to undertake projects with a perspective. I shall not drop everything in Paris to take the risk of losing everything again."

Most other senior scientists who suffered the persecutions and destructions of the past hold the same cautious opinion. Professor Pereira da Silva explains that he "met twice with the fate of losing everything after building a research group; it's already too much." After his first exile in 1964 he agreed to return to a medical school in São Paulo, leaving behind the work on lambda phage on which he was working with Nobel prize winner Francois Jacob. "This was in 1968 when there appeared to be a political lull. But after nine months spent organising a new laboratory in microbial genetics, I and my

How the military banned 'exotic ideas'

The life and death of the Oswaldo Cruz Institute in Manguinhos is typical of the long-lasting unstable interaction between scientific institutions and government in Brazil. The Institute was created at the turn of the century, and its first director, Oswaldo Cruz, received his initial training at the Institut Pasteur in Paris. At that time Rio de Janeiro was a swampy, dirty place, harbouring all kinds of epidemics and chronically affected by yellow fever.

In 1902, Oswaldo Cruz publicized Finlay's discovery that the mosquito *Aedes aegypti* was a transmitter of yellow fever, and coordinated a public sanitation plan to eliminate it. Although this plan succeeded in eradicating the disease within a few years, official resistance was great and Cruz was often ridiculed in the press, and even in Congress, for bringing in "exotic theories".

Two years later, Cruz proposed to institute compulsory vaccination against smallpox. A virulent Antivaccination League immediately emerged, bringing together military men, workers' organisations and old-fashioned medical surgeons — whom the young scientists at Manguinhos described as "always wearing dark suits and using their medical authority to declare quarantines and sign autopsy reports in times of epidemics." Their opposition to compulsory vaccination was in the name of individual freedom of conscience: "vaccination was forced upon us and we may even use force against it. This government must be rejected by the people." Partly as a result of the League's campaign, military cadets rebelled in 1904, in an attempt to bring down the first republican government of Brazil. The government, however, won

the day and the combined medical and military victories led to dramatic increases in funding for medical research at Manguinhos. In the following years malaria was intensely studied; Carlos Chagas identified the *Trypanosoma cruzi* and left his name attached to Chagas disease. Serums and vaccines of all kinds were developed.

"Today," says Professor Ubatuba, "all these achievements seem to be forgotten. That house of science at Manguinhos was destroyed in 1970 when ten of us were forcibly retired. Furthermore, eight of us had our political rights taken away for ten years as stipulated in the Fifth Institutional Act, the act which closed down Congress." The tone had been set by the Health Minister of the military government who had declared, when a new director of the institute took office: "if it is true that there are no frontiers for science, it is also certain that there are frontiers for scientists. The exotic ideas that have infiltrated Manguinhos will be definitely banned . . . Manguinhos of the future will be a busy hive and not what a few wanted, a centre of subversive ideas. The institute will have all the support it needs for its research."

Professor Herman Lent, one of the eight, and former member of the Department of Zoology, recalls in his book *The Massacre of Manguinhos* that the director of the institute had accused its researchers of "conspiring in their laboratories".

A commission of inquiry, headed by a military man, did not find any evidence but it praised the director who "although a poor professional and a poor administrator is a reliable anticommunist". **M.B.**

colleagues were fired once more, and I went back to the Institut Pasteur."

Another decree of exception, the Fifth Institutional Act, was used in 1969 to fire 25 professors from the University of São Paulo and 44 from the Federal University of Rio de Janeiro. Among the latter was physics professor Jose Leite Lopes, who had also gone back to Brazil in 1968, to the Federal University in Rio. He was also reappointed director of the Theoretical Division of the CBPF, the Brazilian Centre for Physics Research, to which he had previously attracted Richard Feynman and C.N. Yang on sabbatical. Federal funding for physics research had improved substantially in the meantime. But in April 1969, Professor Leite Lopes heard on the radio that he had been dismissed from the University of Rio. "The 44 names were read in alphabetical order; you only waited for yours to come up. That was the end."

A few months later, while lecturing in Pittsburgh, he learned of his further

dismissal from the CBPF. "Four colleagues resigned in protest; I got telegrams of support; the SBPC and international societies protested. It was all very comforting at the time. Then there was a lull, to be followed by 12 more firings in 1972 . . .". He now heads the Theoretical Division of the Nuclear Physics Laboratory in Strasbourg, and says "I shall be going back this summer for the first time in ten years, at the invitation of the physics department of my former university in Rio. Beside this I want to see what it is possible to do, and to discuss ideas. It looks from here as if some progress is being made and I am eager to see how far it goes."

Dr Julio Puddles in Paris takes a still more hesitant attitude. "I was among the nine doctors fired from the Faculty of Medicine in São Paulo in 1964; I was also part of the group which prepared a model for the University of Brasilia in 1963. I was one of those found innocent in our trial in

1966, and I fought unsuccessfully for six months to recover my university post. Dr Pudles would like to go to the SBPC meeting in Fortaleza but hesitates because of the cost. "I paid my own way in 1966, all for nothing."

He sees the SBPC as a remarkable society which has consistently combated arbitrariness and shown tremendous dignity in its support of scientists. But he expresses doubts about returning for good. "My bitterness from the past is very deep. The university may let us return, but with the same positions we had in 1964. We'd find there those same people who wrote anonymous letters denouncing us to the military. When you are over fifty, you cannot run the risk of having to look for a job in France again. France accepted me three times in the past, but the new legislation concerning foreigners working in France is very tough and makes it almost impossible for anyone coming anew to stay permanently."

Passport problems stop some from returning

Some Brazilian research institutions have been modified so much over the past fifteen years that exiled researchers cannot imagine going back. Professor Fernando Braga Ubatuba, a pharmacologist working at the Wellcome Foundation near London finds himself in this situation. "The research institute of Manguinhos in Rio de Janeiro where I worked did fundamental research in medical science with departments of microbiology and parasitology of great renown, but today it has been transformed into a mere generator of vaccines. (see box)

"We all rejoiced on New Year's day this year when President General Geisel revoked the Fifth Institutional Act, and I have recently been back for the Congress of the Latin American Society of Pharmacology. But the case of my colleague Professor Haity Moussatche, now teaching in Venezuela, is more difficult, because our government refuses to renew his passport."

The problem of passports always comes up in the interviews. Those unable to obtain one had to take refugee status in their country of residence abroad. "Now the Brazilian government demands that we first renounce our refugee status before it considers whether it will give us passports," says Alice Rivera of the Centre for the Study of Weak Radioactivity in Orsay near Paris. "I would like to go back but I do not believe I could find work in Brazil now, although my only crime is to be married to an ex-deputy whose mandate was annulled when the military took over in 1964." □

Maurice Bazin is a physicist and science journalist specializing in Latin American affairs.

Can we afford to make the fast reactor safe?

The safety of fast reactors is more difficult to guarantee than that of thermal reactors, so the cost of making them safe is likely to be high. **Norman Dombey**, of Sussex University, argues that international collaboration is the only way to proceed.

FAST reactors, in particular the liquid metal fast breeder reactor (LMFBR), differ substantially from thermal reactors; indeed as a physicist I take the view, based on the intrinsic physics of the systems, that fast reactors should be considered as a different genus from thermal reactors.

A discussion from this viewpoint illustrates some of the problems of fast reactor development. In this article I should also like to introduce to non-specialists some of the features characteristic of fast reactors, as this information is not readily available outside the technical literature, and finally, I shall draw some conclusions for fast reactor development generally and for the British programme in particular.

The main physical differences between thermal and fast reactors are given in Table 1, the most important point being the last: thermal reactors are designed to be in their most reactive nuclear configuration. This follows from the presence of a moderator without which the natural or slightly enriched uranium fuel of the reactor would not go critical at all. The corollary of this for fast reactors is that as there is more than one critical mass of fissile material in the core of a fast reactor and no moderator, it is not difficult to envisage a rearrangement of fuel to provide a more reactive configuration. As the reaction goes by fast neutrons, it is possible to attain a prompt critical reaction with a large energy release over a very short time. (About 0.2% of the neutrons in a fast reactor are delayed, i.e. come from radioactive daughter nuclei with various half-lives of the order of one second; it is these delayed neutrons which allow a reasonable control of the fast reactor because the prompt neutrons are captured in about one microsecond. A fast reactor is therefore designed to be just critical including the delayed neutrons, but not critical without them; a prompt critical reaction is one which is critical by prompt neutrons alone.) A large energy release over a short time is called a nuclear excursion and cannot occur in normal types of power-generating thermal reactors.

Another important point in this comparison follows from the absence of a moderator in the fast reactor, which allows the fast reactor core to be much smaller than a thermal reactor of the same power. The power density of a fast reactor is about five times that of a light water reactor (LWR). (We should remember that LWRs themselves have a power density about ten times higher than British gas-cooled reactors.) So the coolant used in a fast reactor must have very high thermal

conductivity and all the present prototypes use liquid sodium.

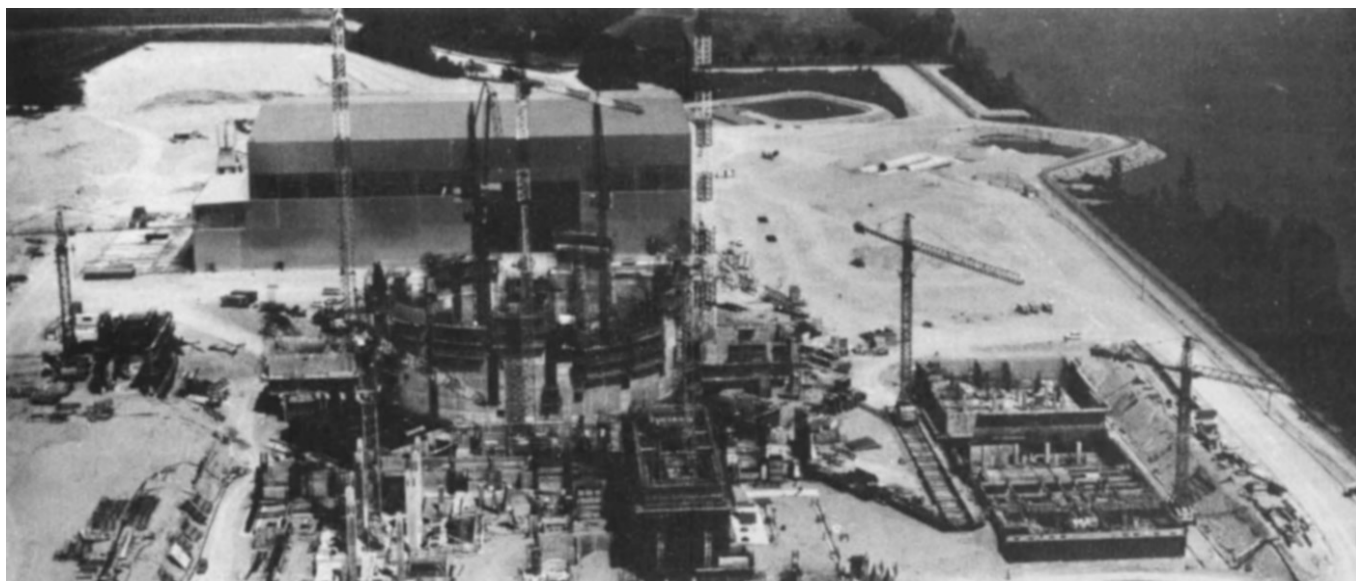
It is a job for the licensing authorities in each country to consider hypothetical accidents and to insist on measures to minimise the risk of accidents, and to minimise and contain their effects if they do occur. The discussion of hypothetical accidents begins with an analysis of the reactivity coefficients of the reactor. These coefficients measure the change in reactivity of a reactor as the physical state of the reactor changes. The important coefficients for safety purposes are those related to an increase in power and temperature of the reactor: if the power or temperature increases, a negative coefficient implies that the reactivity will decrease. As commercial thermal reactors operate in the configuration of maximal reactivity, all nuclear reactivity coefficients in these thermal reactors will be negative. For fast reactors, the reactivity coefficient which allows these reactors to be considered as potentially suitable for commercial use is the Doppler coefficient, which measures the change of neutron reaction rates with temperature in the reactor materials themselves. For uranium 238, neutron absorption increases strongly with temperature and hence, there will be fewer neutrons available for fission, so the Doppler coefficient is large and negative.

The fissile material in the core will be plutonium or ^{235}U ; for the latter the Doppler coefficient is positive in the same temperature range whereas for plutonium it is small. So in a fast reactor of the design envisaged in CFR 1 or Superphenix with about 20-25% plutonium and 75-80% natural uranium (99.3% ^{238}U) in the fuel rods, there will be no overall negative Doppler coefficient.

It is important to emphasise that the Doppler coefficient, although dependent on core geometry, is primarily an intrinsic nuclear quantity. It depends on the nuclear composition of the fuel used and its

Table 1 Primary differences between thermal and fast reactors

Thermal	Fast
natural or slightly enriched (2-3%) uranium fuel	20-30% enriched uranium or plutonium-uranium fuel
less than 1 critical mass	more than 1 critical mass
moderator	no moderator
thermal neutrons, $\frac{1}{10}$ eV	fast neutrons, keV-MeV
most reactive configuration for nuclear reaction	not most reactive configuration for nuclear reaction



Superphenix, Europe's largest (1200 MWe) fast breeder reactor, under construction at Creys-Malville, France.

arrangement in the core; once an acceptable configuration is found, there is no reason why this configuration should not be scaled up to a larger reactor design.

The second most important reactivity coefficient in fast reactors is the sodium void coefficient. Although fast reactors do not have a moderator, the sodium coolant flows in the core and scatters the neutrons, so to some extent it acts like a moderator in slowing down the neutrons. It also absorbs some neutrons. If the core were to overheat, the sodium could begin to boil; the void coefficient then measures the change in reactivity of the core caused by bubbles of sodium gas forming in the liquid sodium flowing through the core.

Here there are two competing effects. First, sodium gas would absorb and scatter fewer neutrons than liquid sodium, so there would be more neutrons of higher energy and the reactivity would tend to increase. Second, the presence of sodium bubbles would increase the probability of neutrons escaping from the core through the bubbles, so this tends to decrease reactivity.

The first effect leads to a positive void coefficient and is proportional to the volume of sodium in the core, whereas the second effect is an edge effect which is proportional to the effective surface area of the sodium. So for small reactors, where all the sodium can be taken to be near the surface, the edge effect wins and the void coefficient is negative. This was the case for the old 15 MWe Dounreay Fast Reactor. As the reactor gets larger, the volume effect will begin to dominate and the present prototypes such as the Prototype Fast Reactor (PFR) or Phenix have a positive void coefficient, albeit small. The planned CFR 1 or Superphenix, however, will have a large positive void coefficient, about five times larger than that of the present prototypes. So scaling up from a 250 MWe prototype to a 1300 MWe 'commercial' reactor is not simple, because the problems connected with voiding get much worse.

In addition to a comparison of reactivity coefficients, three other factors need to be considered in any comparison of fast reactors with thermal reactors: the fuel-coolant interaction, other non-nuclear problems arising from the use of liquid sodium as coolant, and the general problem of recriticality.

The fuel-coolant interaction is not a nuclear effect: it is the potentially explosive interaction arising when a molten metal comes into contact with a liquid at a temperature much above the boiling point of the liquid. It is well known in the steel industry from incidents where molten steel has come into contact with water, and it is also a problem inherent in water-cooled thermal reactors, as it has been known for a piece of hot fuel rod to melt and interact explosively with the surrounding water. For LWRs, however, the fuel-coolant interaction is less critical than in large LMFBRs because there is an overall negative coolant void coefficient.

The best known difference between thermal reactors and the LMFBR stems from the difficulties of using liquid sodium as coolant in the LMFBR. Sodium is, of course, highly corrosive and interacts vigorously with both air and water on contact. All those large prototypes at present operating (PFR, Phenix and the Soviet BN-350) have been plagued by sodium leaks.

In fast reactors there are also recriticality effects to consider. As the core is not in its most reactive configuration and there may be hundreds of critical masses of plutonium in it, a disruption of the core may lead to a much more reactive configuration; for example, a coolant channel gets blocked, the sodium boils, the reactivity increases because the void coefficient is positive, the cladding of some fuel rods melts and pieces of fuel containing plutonium fall and form a molten mass of plutonium which if large enough can then become prompt critical

leading to a rapid release of energy.

The analysis of the energy release and the pressures created by such hypothetical core-disruptive accidents is being pursued in all the countries engaged in fast reactor technology. Enormously complicated computer programs are being prepared to simulate these conditions. Whether or not the present programs are reasonably accurate in describing fast reactor accidents, it is clear that the role of negative reactivity coefficients will be to limit the energy release whereas positive coefficients will enhance it. Thus for a reactor of the size of PFR or Phenix with uranium-plutonium fuel, the energy release for a core-disruptive accident of the type described is not expected to be larger than 400 megajoules (equivalent to about 200 lb of TNT). But in a reactor the size of CFR 1 or Superphenix, the sodium void coefficient is much larger and positive so the energy release for a similar accident could be 2000 megajoules (1000 lb of TNT).

Now the point is not that fast reactors, or large fast reactors, will blow up — it is the job of the licensing authorities after all to ensure the safety of such systems. But the licensing authorities will insist

- on minimising the risk of an accident
- on limiting the damage to the reactor core in the event of an accident
- on maintaining the integrity of the primary reactor system, even in the event of a core-disruptive accident leading to a large energy release
- even in the worst case of an accident breaking the primary system, that radioactive materials are contained.

These are the four lines of assurance of the US authorities, but the practice of the British Nuclear Installations Inspectorate should be similar. The point that I want to make is that measures to deal with the safety and other engineering problems specific to fast reactors will add appreciably to the capital costs of fast reactors compared with thermal reactors of a

similar size. Yet the goal of a commercial fast reactor system is to produce cheaper electricity than other systems such as thermal reactors. Fuel costs play a minor role in the total cost of nuclear electricity, so that fast reactors will only be competitive with thermal reactors if their capital costs are comparable (unless the price of uranium were to increase dramatically). The special safety requirements of fast reactors and the difficulties of using sodium in LMFBRs both suggest that the capital cost of a LMFBR will be much higher than a comparable thermal reactor. Furthermore, as has been indicated, the safety problems get worse for large fast reactors. It may be thought that my comments are overly pessimistic or overly theoretical — I am after all a theoretical physicist. But the one case study available on the capital costs of fast reactors does indeed bear out my conclusions.

The prototype German fast reactor SNR-300 has increased in cost (at constant 1972 prices) from the initial estimate of DM300 million at the time the reactor was authorised to the most recent estimated cost DM1223 million in October 1975 (O. Keck, University of Sussex Doctoral Thesis, 1977). These are not the final figures and the cost escalation is therefore by at least a factor of four in real terms. Keck also demonstrates that the initial cost estimate of the SNR-300 was in no way based on any evidence about the likely capital costs of fast reactor systems. The estimate was simply a number which the fast reactor proponents thought to be reasonable in view of the likely cost of a thermal reactor system of comparable size — I suspect that the figures we have heard from the AEA about the likely cost of CFR 1 have been arrived at in a similar way. Keck now estimates that the capital cost of the SNR-300 is five times that of a comparable LWR system ordered at the same time.

The importance of this case study is that the SNR 300 is the first and so far the only prototype fast reactor which has been built as if it were a commercial reactor; that is to say it has been built specifically with the goal of satisfying both the utilities and the licensing authorities. Therefore the utilities and the licensing authorities have been consulted from the early design stage in 1969. Keck says "After the designs were submitted drastic alterations became necessary as a result of the safety and environmental provisions demanded by the licensing authorities and substantially increased the costs of the plant... The most severe licensing requirement related to hypothetical accidents. The safety philosophy of the original design placed the emphasis on measures to prevent a core meltdown and a subsequent hypothetical nuclear excursion, and it was thought that this accident could be made sufficiently unlikely to avoid measures accommodating its consequences. The licensing authorities, however, not only required more stringent measures for prevention of this accident,

France minimises risk of 'worst accident'

WHEN construction was authorised in May 1977 of Superphenix — the fast breeder reactor now being built in south-east France — the licensing authority stipulated that it should be built to meet certain safety specifications. One was that the double-walled reactor vessel, the lid and the dome housing the reactor, should be able to withstand the accidental release of 800 megajoules of mechanical energy. This was the energy which was believed might be released in the 'worst possible accident', that of criticality followed by a sodium explosion.

More recent studies by the French atomic energy authority and others (reported in *Le Monde*, 5 July) however, have concluded that the likelihood of such an accident happening is much lower

than had previously been thought: 1 in 10^8 instead of 1 in 10^6 . Three independent rod systems, the studies conclude, lower the probability of criticality. Even if the reactor did go critical, then it is highly unlikely that the energy released would be 800 MJ.

The recent studies have also shown, however, that the reactor buildings would be unlikely to withstand 800 megajoule explosions. (It is not ever clear precisely what maximum energy they could withstand.) The question therefore remains whether, given the reduced probability of an 800 megajoule explosion, the licensing authority will still insist that the reactor housing be built to withstand such energies — which would mean a substantial increase in cost. □

but also stipulated that the impact of a nuclear excursion should be safely accommodated by the reactor vessel and the containment. . . .

"The demands of the licensing authorities not only increased the costs of the prototype plant, but also necessitated a lot of additional research and development in industry and government laboratories, which added considerably to the costs of the fast breeder project. For some items, the licensing requirements even went to the limits of technical feasibility, and imposed heavy tasks on scientists and engineers in industry and government laboratories."

So on the basis of this case study, there is every reason to believe that the licensing requirements for the fast reactor, arising specifically from the physics problems I have outlined, will cause an appreciable increase in capital costs relative to thermal reactor systems. Furthermore, the situation will get worse for larger fast reactors such as CFR 1 and Superphenix.

The relative higher capital costs of fast reactors compared with thermal reactors must be weighed against the prospective future price of uranium. In the words of *The Times Mining Correspondent* (*The Times*, 30 October 1978) "colossal new discoveries of uranium" have been made in the past three years in Australia and Canada. Therefore "prices are unlikely to go up faster from their current \$42 a pound than inflation warrants". So there can be no urgency about developing a commercial fast reactor for economic reasons; it would be more cost effective to spend money on improving the present designs of thermal reactors. Although the AEA talk about a commercial fast reactor, there is no reason at present to use the term commercial. There still remains an enormous amount of basic work to be done on these systems before they can be regarded as a safe, sure and reasonably-priced source of electricity.

My final point is that fast reactors are too hard for us in Britain working alone, since we have neither the resources in

money nor in people to do the job by ourselves. The greatest part of the UKAEA's budget and most of its qualified scientists and engineers since the mid-1960s have been committed to the fast reactor but, nevertheless, we cannot match the spending and manpower committed in the United States on fast reactor systems.

The UK is favourably placed for energy until the end of the century, while fast reactors cannot hope to be commercial until next century. A modest programme of research and development is indicated which does not strain the UK's resources, so that it is in a position to benefit from any future exploitation of fast reactors next century. The problem is similar to that in the field of fusion power and the solution is similar: there should be a common European programme of research and development, similar to JET and to the programme of research in elementary particle physics based at CERN in Geneva. If it is too late for the UK to join in the Superphenix project, it should indicate its willingness to join the next reactor after that. With its continuing experience of PFR at Dounreay and its large and assured stock of plutonium it can expect to be welcomed as a partner. I agree with Lord Flowers (*Nature* 264, 496; 1976) that 'fast reactor developments is likely to be beyond our resources. It seems to me essential that if we propose it at all we should do so on a European basis'.

So it does not make sense for the UK to build CFR 1. On the other hand there is scope for being involved in planning the reactor to be built as a European collaboration (perhaps even the US and Japan would also be interested in participating) after Superphenix. The considerations I have outlined here would suggest that a sensible size for a LMFBR which avoids the problems connected with a large positive sodium void coefficient and which may have commercial potential next century would be about 500 or 600 MWe. PFR, Phenix and SNR-300 provide a sensible basis for the design of a reactor of this size.

news and views

The oceanography of fjords

from David M. Farmer and Herbert E. Huppert.

FJORDS, inlets or sea lochs are deep, twisting arms of the world's oceans, generally formed by glacial erosion. In sharp contrast to the depth of the main basin — up to 1700 m — most fjords have a shallow sill at their mouth, of between 1 and 10² m, and sometimes with second, third and further sills. All fjords are influenced by the tides, the prevailing winds and fresh water input from rivers. Some, particularly those in the Arctic, are covered with ice for part of the year. Many people live along their shores, especially in Scandinavia, and have come to rely on the fjords for water, food, the disposal of waste and recreation. All these demands are interconnected, so that the ill-considered construction of a hydroelectric scheme, for example, will modify the water circulation, which in turn can decrease the production of herring in the fjord and of codling on the continental shelf. Man's intervention can also seriously disturb the millions of juvenile salmon which use fjords as highways on their seaward migration and depend throughout their journey on food produced in the fjords.

Scientifically, fjords provide a relatively simple system for investigations which can also contribute to knowledge of the open ocean. All these aspects were borne in mind at a recent NATO Advanced Research Institute on the physical, biological and chemical oceanography of fjords.*

G.L. Pickard (University of British Columbia) and B.R. Stanton (New Zealand Oceanographic Institute) have compiled a detailed description of the water characteristics in Pacific fjords. In contrast to the open ocean, their density structure is controlled almost entirely by salinity (rather than temperature). In Pacific fjords the salinity almost always increases with depth, whereas the temperature and dissolved oxygen generally decrease slightly. The river run-off near the head of each fjord, although very variable, can make an important contribution to the circulation. Fjords with

high river run-off have well-mixed layers up to 10 m in depth, and there tends to be some movement in the deep water. The Alaskan and Chilean fjords with glaciers flowing into them, in contrast to those without, tend to have a positive dissolved oxygen anomaly at depth and a stepped salinity structure. This can be explained in terms of the predominantly horizontal flow, in a series of layers, of the glacier melt water and the extra oxygen contained in the ice as suggested recently (Huppert & Turner *Nature*, 271, 46; 1978). Although morphologically similar to Pacific fjords, Scandinavian fjords typically experience smaller tides and this may have important consequences for the types of mixing process which dominate. A further distinction is that New Zealand fjords are short, have relatively deep sills and their circulation, in contrast to other fjords, tends to be set by the oceanographic conditions along the coast.

R.R. Long (Johns Hopkins University, Baltimore) felt that rare but severe disturbances, such as bad storms, could be responsible for a considerable amount of the vertical mixing and affect the circulation in fjords and in the deep ocean. The concept that rare, large, inputs might be considerably more influential than the inputs usually observed, was much discussed, but the final consensus was that the available data from all sources were not sufficient to make a definitive statement.

The renewal of deep water, due to the occasional flow of relatively heavy water over the sill into a fjord may occur over a range of time-scales — as infrequently as only once every 7 years in one case — in response to a combination of tidal range, strength of the off-shore winds, amount of river run-off and interaction of the flow produced by these effects with the major sills (A. Edwards, Dunstaffnage Marine Research Laboratory, Scotland). In between renewal, diffusion and mixing slowly decrease the salinity at depth, thus helping to promote the next renewal. Mixing may be the result of the breaking of internal waves and of turbulence generated by the tidal flow past the boundaries, although the magnitude of both these mechanisms is uncertain. D. Dyrssen (Göteborg University, Sweden) drew

attention to the practical importance of deep-water mixing in connection with pollution. Deep water exchange is also crucial in determining the amount of available oxygen, which in turn produces changes in the distribution of species and alterations in feeding behaviour.

From the results of the JASIN (Joint Air-Sea Interaction) experiment R. Pollard (Institute of Oceanographic Sciences, UK) pointed to three mixing processes believed to be relevant to the oceanic thermocline and possibly to the halocline in a fjord. First, there is the instability, wave breaking and mixing due to shear, which Pollard believed to be infrequent and unimportant in the oceanic thermocline. Second, there is the motion along isopycnals, as for example, across fronts. Third, there is the motion across isopycnals due to double-diffusive effects. The importance of the last effect is still controversial, but data from the open ocean continue to affirm its influence and Pollard discussed additional confirmation in data taken by M. Gregg (University of Washington, Seattle). Laboratory experiments by B.R. Ruddick and J.S. Turner (*Deep-Sea Res.*, in press) indicate that double-diffusive effects may also play a part in mixing across fronts. Pollard then pointed to processes believed to be relevant in the mixed layer: the generation of Kelvin-Helmholtz billows, with subsequent mixing, surface wave breaking and Langmuir circulation due to interactions between surface waves and shear in the water. Various theoretical models have been proposed to predict features of the upper mixed layer, but it seems that the models are more sophisticated, though less realistic, than warranted by the data.

One of the most comprehensively observed North American fjords is Knight Inlet in British Columbia and an impressive array of phenomena have been documented there. Acoustic soundings indicate that there is a large amplitude internal response to the tidally forced flow

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* The Fjord Oceanographic Workshop was held at the Institute of Ocean Sciences Patricia Bay, British Columbia from 4 - 8 June, 1979. The proceedings are to be published by Plenum in their Marine Science Series, edited by D.M. Farmer and H.J. Freeland.

over the sill. At the beginning of the ebb tide, the flow separates behind the inner sill and there is a large amplitude periodic disturbance on the shear layer emanating at the sill crest, at a depth of approximately 60 m. As the tidal flow increases, a large-amplitude, stationary lee-wave train, or more usually an internal hydraulic jump, is set up in the stratified layer in the lee of the sill. Such an event is shown in the illustration along with the laboratory simulation. The strong flow associated with the accelerated flow down the lee face of the sill suppresses the separation, in agreement with recent theoretical and laboratory experiments of P. W. M. Brighton (Ph.D. Thesis, University of Cambridge). As the tide slackens, waves propagate upstream, since their phase velocity is now larger than that of the opposing tidal flow. This is the same phenomenon as documented by acoustic measurements in the vicinity of Stellwagen Bank in Massachusetts Bay (Haury, Brisco & Orr *Nature* **278**, 312; 1979), a model of which was investigated in the laboratory (Maxworthy *J. geophys. Res.* **84**, 338; 1979). From measurements taken by the small manned submersible Pisces IV, A. Gargett (IOS, British Columbia) found that the wave train in Knight Inlet has an amplitude of 10 m, is highly turbulent and has a very strong down-ward velocity at its front. The flood tide may set up a similar train of waves, which propagate

downstream as the tide slackens, but they are of smaller amplitude and possibly of different character because of the asymmetric geometry of the sill.

Long discussed refinements of his two-layer frictional model for the circulation in a fjord, presenting an updated parameterisation of the friction to allow for a better comparison with data from Knight Inlet. Nevertheless, a consequence of the model, that the Froude number at the mouth be 1.0 (the critical value) is not reflected in recent data taken in Knight Inlet, which indicates that the value is close to 0.3.

Some of the many practical problems in fjords were skillfully described by Scandinavian workers. Careful consideration of the energy exchange between the barotropic and baroclinic tidal response over a sill indicates that some fjords have constrictions which make them close to resonant. This suggests that relatively small modifications of the constriction, such as altering the depth of the sill or placing a floating bridge or barrier over the sill, may bring the fjord closer to resonance. This would increase the tidally-generated internal response and lead to enhanced mixing.

The siting of hydropower installations and the depth of the fresh water discharge into a fjord can have enormous influence on the water characteristics; in particular, a fresh water layer, sufficiently deep and

cold, on the top of a fjord can promote undesirable ice cover throughout the winter. T. Carstens (Norwegian Institute of Technology, Trondheim) explained that submerging the output and introducing compressed air into it enhances the mixing in the upper layers and increases the salinity at the surface, thereby inhibiting ice formation. T.A. McClimans (Norwegian Institute of Technology, Trondheim), described models for the point release of a fresh water supply into a fjord, incorporating tidal effects, which have been successfully used in a number of situations.

A novel technique for studying the circulation in the Clyde Sea Area fjord involving the use of radioactive pollutants from Windscale was presented by I.G. McKinley (University of Glasgow, Scotland). Caesium-134 and 137, which have different half lives (2 and 30 years), provide an ideal tracer pair for studying vertical diffusion, sedimentation rates and other exchange processes. The caesium was detectable as far away as off Northeast Scotland, suggesting that this procedure may be ideally suited for ocean circulation studies elsewhere. □

Changing cometary orbits

from David W. Hughes

Two principal mechanisms are available for changing the orbit of a comet. The first is gravitational and relies on the proximity of a planet to perturb the comet's path. The change in the reciprocal of the semi-major axis of the orbit then suffers a random walk which is approximately proportional to the square root of the number of returns the comet makes to the inner Solar System. The second is non-gravitational and is due to the fact that gases given off by the cometary nucleus are not emitted symmetrically in all directions. The importance of this second force was first realised in the early 1820s when Comet Encke consistently passed perihelion 2.5 h before the predicted time.

Imagine an icy conglomerate cometary nucleus which is not rotating. The maximum emission of the sublimating volatiles will occur on the sunward side, around the subsolar point. This jet of gas will effectively exert a force on the nucleus in the direction away from the Sun. Now if the nucleus rotates and if there is a time lag between the absorption of the solar radiation by the dusty surface of the nucleus and the sublimation of the cometary ices, then the effective force is not radial; this force will have a transverse component along the orbit of the comet and also a normal component perpendicular to this orbit. Marsden, Sekanina and Yeomans (*Astron. J.* **78**,

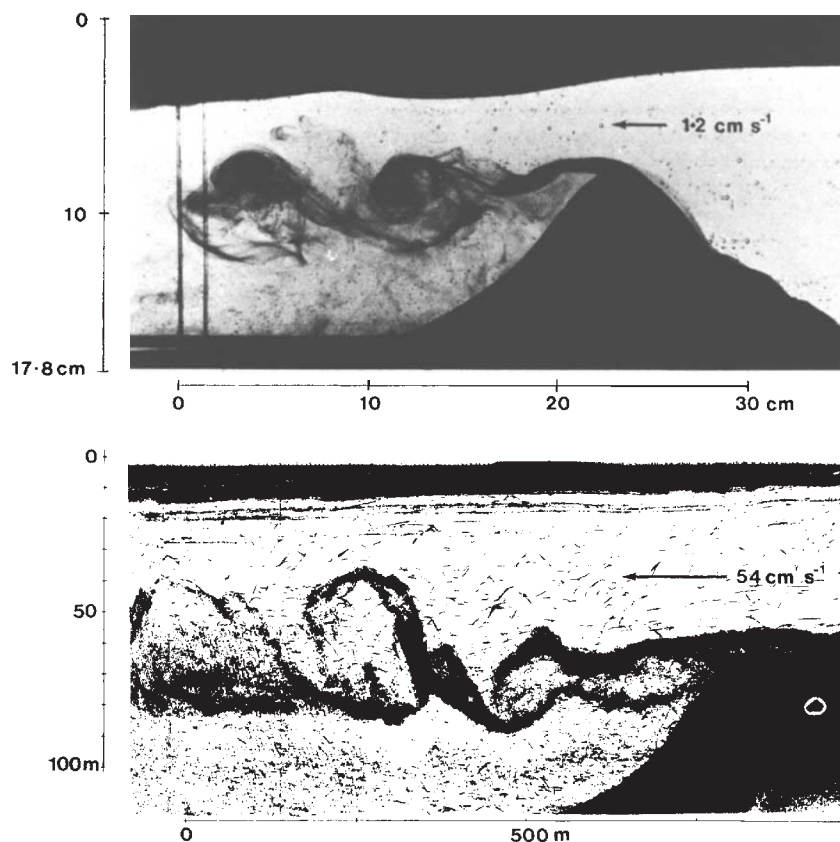


Fig.1. Tidally-generated flow in the laboratory (bottom) and Knight Inlet, British Columbia (top), showing the effects of flow separation and large-amplitude billows.

211; 1973) found that the radial force was typically 10 times the transverse force and that the normal component was either zero or undetectable. This implies that the lag angle (the angle between the solar direction and the line from the centre of the nucleus to the maximum emission point) is on average about 6° . Such a low value indicates that the nucleus is either spinning slowly, with a period of 24 h or more, or that the spin axis is nearly perpendicular to the orbital plane, or that both these conditions are operative.

Interestingly there seem to be as many comets with positive transverse forces as with negative ones. So direct rotations (like the Earth's) and retrograde rotations are equally probable.

The effects of these transverse forces are also rather interesting and they have been investigated by Paul R. Weissman (Jet Propulsion Laboratory, California) in a recent edition of *The Astronomical Journal* (84, 580; 1979). If the cometary nucleus has a direct rotation, the transverse force increases the energy and the angular momentum of the orbit and both the perihelion distance and the aphelion distance get larger. The comet moves away from the Sun. The opposite occurs for retrograde rotation. Now planetary perturbations produce a random walk variation in orbital parameters whereas non-gravitational forces are much more effective. Assuming that the spin axis direction does not vary, the value of the reciprocal of the semi-major axis undergoes regular, unidirectional steps and the total change produced in this quantity is simply a function of the number of times the comet passes close to the Sun.

Non-gravitational forces are responsible for changing the orbits of many of the short-period comets. Weissman suggests for example that they have decreased the semi-major axis of the periodic comet Encke and have pulled it away from the influence of Jupiter. This effect can thus greatly increase the inner Solar System lifetime of these short-period comets and there is the possibility that they could lose all their ices without violent disruption and so evolve into Apollo or Amor-type asteroids. The action of non-gravitational forces on long-period comets is much more difficult to assess simply because these are only seen at one apparition.

Weissman also considers the Kreutz family of Sun-grazing comets. The change in the reciprocal of the semi-major axis, a , that these non-gravitational forces can produce, increases strongly as the perihelion distance gets smaller. The Kreutz family have perihelia in the region between 0.005 and 0.009 AU, well within the solar Roche limit. Typical values of $\Delta(1/a)$ lie in the range 10^{-3} to 10^{-1} AU $^{-1}$ and can easily change the aphelion distance from around 4×10^4 AU (well within the Oort cloud) to

their present value of about 100 AU, in two or three returns.

Of course there are still many problems requiring solutions. Do the spin periods and spin axis directions of cometary nuclei vary with time? What made them spin in the first place? Are the spin axes directions randomly orientated in space and if so why? F.L. Whipple (*IAU Colloquium* 39, 25; 1977) reminds us that the cometary nuclei are irregularly shaped objects that can lose up to 1% of their mass at each perihelion passage. Also the mass loss is irregular and often occurs disruptively. These factors can all lead to a change in the spin axis direction and the spin rate.

Another mechanism that can change the spin rate has been discussed recently by R.L. Hawkes and J. Jones in *The Monthly Notices of the Royal Astronomical Society* (185, 727; 1978). They consider the effect of micrometeoroid ablation on the spin rate of the meteoroids responsible for the radio and visual meteors seen in the Earth's atmosphere. Particle impacts on an irregular body will produce a change in the angular momentum and the total angular momentum will increase in a random walk fashion. Even though on average there will be as many collisions causing increases in the spin rate as there are causing decreases, there will be an incomplete cancellation due to the statistical fluctuations of the opposing torques. According to Hawkes and Jones the meteoroids spin up until rotational energy becomes comparable to the translation energy. The meteoroids would then be spinning at about 1000 revolutions per second indicating that comets with their spin periods of many hours have a long way to go. □

Progress in thalassemia research

from David G. Nathan

INVESTIGATIONS of normal haemoglobin have paved the way for understanding the anatomy and physiology of the human genome and research into Cooley's anaemia has been well in the vanguard. At the fourth symposium on Cooley's anaemia,* an international group of clinicians and basic scientists met to review the remarkable progress made in understanding, treatment and prevention of this inherited disease.

Cooley's anaemia, or the β -thalassaemia syndrome, together with the various α -thalassaemia syndromes are the result of unbalanced synthesis of globin chains secondary to decreased production of functioning mRNA. This mRNA deficit is sometimes due to specific gene deletions. More commonly, the structural gene is present but ineffectively contributes to

globin synthesis. Application of restriction endonuclease gene mapping techniques to normal DNA and to DNA bearing globin gene deletions by R. Bernard, R. Flavell and R. Williamson (University of Amsterdam and St. Mary's Hospital Medical School London) and J. Mears and A. Bank (Columbia University, New York), has provided a physical map of the γ - α - δ - β globin regions in chromosome 11, one that spans more than 40 kb (kilobases). The intergenic distances are now established. Maps of several different deletions in this region, all of which cause complete absence of β globin synthesis (β^0 thalassemia) have been provided by B. Forget (Yale University), T. Maniatis (California Institute of Technology; also *Nature*, 279, 598; 1979), Mears and S. Orkin (Children's Hospital Medical Center, Boston). They range from a 0.6 kb deletion at the 3' end of the β gene to much larger deletions that cause either $\delta\beta$ thalassemia or hereditary persistence of fetal haemoglobin. In the latter diseases the known deletions include one of at least 30 kb encompassing all of the DNA from the area immediately 3' to the γ gene through to the 3' end of the β gene and another of at least 13 kb encompassing the 3' half of the δ gene through to the 3' end of the β gene. Whether a firm relationship exists between the extent of gene deletion 5' to the β , and γ chain synthesis is uncertain from the few cases studied to date.

Two laboratories have reported successful cloning of the β^+ thalassemia genes. So far molecular lesions clearly responsible for disturbances in transcription or mRNA processing have not emerged. Forget reports no abnormality in the 5' end of the larger β intron (intervening sequence). Unambiguous interpretations of these clones may be difficult because the data already suggest substantial normal molecular heterogeneity in and around the 5' regions of the β gene. The search for a molecular basis of non-deletion forms of thalassemia will require painstaking sequencing of long stretches of DNA and use of functional systems for detecting gene expression.

Chang and Y.W. Kan (University of California, San Francisco) have clearly ascribed one form of β^0 thalassemia to a nonsense mutation at nucleotide codon 17. The nonsense-containing mRNA can be translated in a cell-free system if a suppressor tRNA^{Ser} is added. This is the first description of a nonsense mutation in man.

The α -thalassaemias have been investigated by S.E. Embury (University of California, San Francisco) and Orkin. 'Non-deletion' forms of α -thalassaemia genes are commoner than formerly appreciated and single deletions of one of

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*The Fourth Symposium on Cooley's anaemia, co-sponsored by the New York Academy of Sciences and the Cooley's Anaemia Foundation Incorporated, was held in New York on 21-23 May.

the pair of duplicated alpha genes is very common in blacks accounting for the rarity of α -thalassaemia-induced hydrops fetalis in that race.

Kan, B. Alter (Children's Hospital Medical Center, Boston), A. Cao (University di Cagliari, Sardinia) and D. Loukopoulos (University of Athens) all reported that prenatal diagnosis of β -thalassaemia by fetal blood sampling is now an established procedure. Endonuclease mapping of fetal fibroblast DNA can also be used in the deletion forms of β -thalassaemia, in certain West Africans with sickle cell anaemia and α -thalassaemia.

R. Propper (Boston), A. Nienhuis (National Institutes of Health, Bethesda) and S. Piomelli (New York University Medical Center) reviewed therapeutic advances in which new approaches to transfusion with young red cells and continuous subcutaneous infusion of deferoxamine can achieve negative iron balance in these repeatedly transfused patients. New approaches to computerised radionuclide cardiac scanning will probably permit better evaluation of the effects of this treatment on cardiac function.

Whether the long-sought goal of increased fetal haemoglobin synthesis can be achieved in patients with β -gene defects was debated somewhat inconclusively by G. Stamatoyannopoulos (University of Washington, Seattle), Nienhuis and Nathan. Normal erythroid stem cells do form differentiated colonies that vary in their capacity to express γ genes. One human erythropoietic cell line (K562) derived from a patient with chronic myelogenous leukaemia was reported by T. Rutherford and D. Weatherall (The Radcliffe Infirmary, Oxford). When stimulated with haemin, this cell line exhibits α , ζ , ϵ , and γ gene expression (*Nature* 280, 164; 1979). W. French Anderson (National Institutes of Health) reported progress in cell fusion studies in that hybrids formed by human fibroblasts and 2-S mouse erythroleukaemia cells produced human β , but not γ mRNA. A specific positive regulator is apparently present in these interesting hybrids.

It seemed clear to all participants that advances in haematopoietic stem cell culture and in globin gene cloning may lead to marrow autotransplantation experiments in animal models in which new functioning globin genes may be incorporated into replicating stem cells and globin chimaeras created in the laboratory. Whether these techniques will be applied in man will await much more refined methodology. In the meanwhile, prevention by prenatal diagnosis and improved therapy are available. Progress at the bench and bedside is indeed exciting in this fascinating field. □

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Quark fusion

from Peter Landshoff

EXPERIMENTS in which beams of high-energy electrons, muons or neutrinos are scattered on a hydrogen target give good, though indirect, evidence that the proton is made of quarks. The evidence is indirect because no free quarks have yet been seen, and indeed most high-energy physicists have come to believe that quarks can only occur in bound states such as protons or π -mesons. For this reason, it is valuable to have other types of experiment that confirm the quark structure and give more information about it. In the experiments that are most straightforward to interpret, a lepton pair is produced in the collision of a proton or pion beam with a hydrogen target, or with some heavier nucleus. The lepton pair may be either an electron and a positron, e^+e^- , or a muon pair $\mu^+\mu^-$.

It was experiments of this type that made the important discoveries of the J/ψ and T resonances. The T are responsible for the structure near 10 GeV in the data shown in Fig. 1 from the Columbia-Fermilab-Stony Brook collaboration, where the production cross-section is plotted against the invariant mass, m , of the lepton pair. Attention is now turning to a more accurate exploration of the continuum, that is, to values of m not near a resonance. In 1970, S.D. Drell and T.-M. Yan predicted that, in experiments with high-energy beams, dilepton production in the high-mass continuum would take place through quark fusion. The Drell-Yan mechanism is best visualised in the centre-of-mass frame, in which the beam particle and the target both move towards each other with large momentum and collide. At that time, it was already known, from experiments in which high-energy electrons scattered on protons, that a substantial part of the momentum of a fast-moving

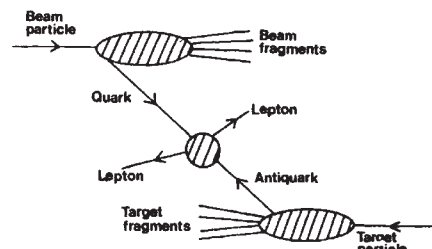


Fig. 2. The Drell-Yan mechanism, in which a quark and an anti-quark fuse to form a lepton pair.

proton is carried by three quarks, known as its valence quarks, but that in addition the proton contains a large number of slow-moving pairs of quarks and antiquarks. In the Drell-Yan mechanism, a quark from one of the two initial colliding particles fuses with an antiquark from the other, and the energy of the quark-antiquark pair then materialises again at the lepton pair (Fig. 2).

The experimental results agree well with this picture. In particular, the proton-beam data verify that the production cross-section $d\sigma/dm$ obeys a scaling law. This is predicted from the fact that quarks and antiquarks have very small radius, or maybe are even pointlike. This implies that the production must be essentially independent of any fixed parameter that carries dimensions, and, since $d\sigma/dm$ has dimension (mass)³ in units where $\hbar = c = 1$,

$$\frac{d\sigma}{dm} = \frac{1}{m^3} F(\tau).$$

Here F is a dimensionless function of the only dimensionless variable associated with the production, $\tau = m^2/s$, where $\sqrt{s}$ is the total energy measured in the centre-of-mass frame. The test of scaling is shown in Fig. 3, where it is seen that $m^3 d\sigma/dm$ plotted against τ is independent of beam energy, or equivalently of the centre-of-mass energy $\sqrt{s}$. Data at rather higher centre-of-mass energies are now beginning to come from the CERN Intersecting Storage Rings, and they also seem to be in agreement with scaling. In the case of π -meson beams, scaling sets in only at rather larger values of m ; why this should be is not understood.

The proton-beam data agree well with quantitative calculations of $F(\tau)$. These calculations use as input the momentum distributions of the quarks and antiquarks within the proton, as measured in the electron, muon and neutrino scattering experiments. However, these experiments determine well the momentum distributions of the valence quarks, but much less well those of the antiquarks, and the dilepton production experiments actually give the best determination of the antiquark distributions. Because of its very short lifetime, the π -meson cannot be used as a target, and so there is no information

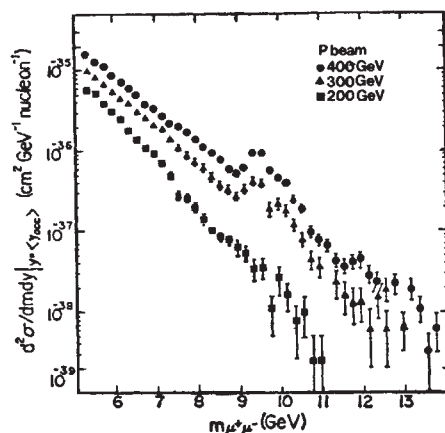


Fig. 1. The invariant-mass spectrum of $\mu^+\mu^-$ pairs produced by high-energy proton beams colliding with a heavy target. The structure around 10 GeV is associated with the T resonances (Columbia-Fermilab-Stony Brook). From a talk given by John Dowell at an Institute of Physics meeting in April, 1979).

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about its structure from electron, muon or neutrino scattering. But muon-pair production from pion beams, when interpreted in the Drell-Yan picture, agrees well with theoretical prejudices about the momentum distributions of quarks within the pion. Quarks are known to have spin $\frac{1}{2}$ and at values of the invariant mass m high enough for scaling to have set in, the angular distribution of the produced muons is found by the Chicago-Illinois-Fermilab collaboration to correspond with that predicted from the fusion of a pair of particles of spin $\frac{1}{2}$.

One surprising property of the Drell-Yan continuum was discovered by the Columbia-Fermilab-Stony Brook collaboration. It was expected that the dilepton system would emerge from the reaction with its total momentum in a direction closely parallel to the incident beam momentum, but it is found that rather often this is not so. Theorists would like to interpret this as a natural consequence of quantum chromodynamics. This is believed to be the quantum field theory associated with the strong interactions that hold quarks and antiquarks together within protons and pions. Just as in quantum electrodynamics the electromagnetic force is transmitted by a particle, the photon, so in quantum chromodynamics the strong force is

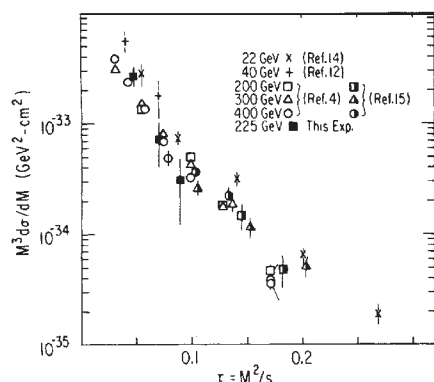


Fig. 3. Test of scaling: plots of $m^3 d\sigma/dm$ for various energies of the incident proton beam (Chicago-Illinois-Princeton). From Anderson *et al.* *Phys. Rev. Lett.* **42**, 944; 1979).

thought to be transmitted by a particle called the gluon. The bremsstrahlung of gluons by the quarks and antiquarks before they fuse can appreciably change their directions of motion, and so may account for the large transverse momentum component of the dileptons. If this is the correct explanation, the gluon bremsstrahlung will have other consequences that can be checked in accurate experiments, including small but well-defined deviations from scaling and from the simple expectations for the angular distributions. The strong interest of both experimentalists and theorists in the Drell-Yan mechanism seems likely to continue. □

A new form of electron diffraction by atoms

from C. B. Lucas

STUDIES of electron scattering by free atoms have now over 50 years of history, so it is surprising that a new diffraction phenomenon has just been discovered by Geiger and Morón-León (*Phys. Rev. Lett.* **42**, 1336; 1979).

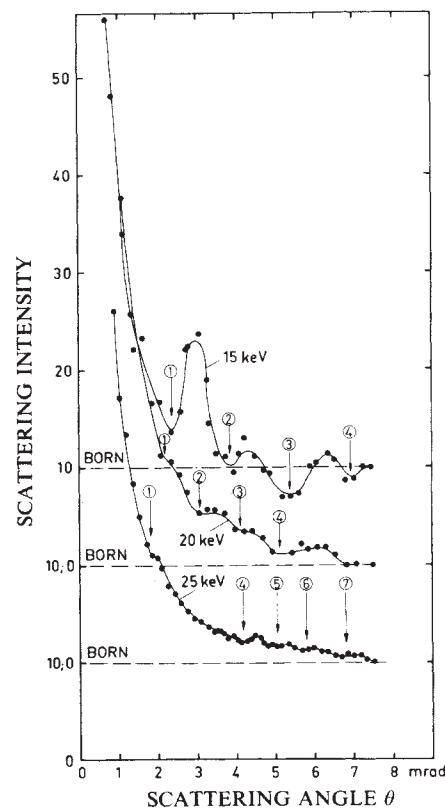
The first experiments which showed that electrons have a wave nature and so undergo diffraction effects were those of Davisson and Germer in 1927, using a crystalline target. These experiments overshadowed the studies of electron scattering from free atoms which started at about the same time. As Bullard and Massey stated in 1931: "It will be seen that the . . . curves exhibit general similarity to those representing the intensity of light scattering from small spheres . . . However the analogy must not be pushed too far as the electron wavelength varies very rapidly in the region of the atom."

In so well-established a field, little general interest is aroused by the majority of current publications on elastic electron scattering. Recent progress has mainly been directed towards increased accuracy of experimental data and increasingly sophisticated numerical calculations. Though great interest was aroused by the discovery of resonance phenomena, which caused narrow structure to be seen in the scattered intensities, certainly the most outstanding discovery of recent times was also made in the 1960s — that electrons scattered from heavy atoms are spin-polarised. This confirmed predictions made by Mott in 1929, using the relativistic form of the Schrödinger equation, which had been introduced by Dirac only a year earlier. Even this exciting discovery did little to raise the prestige of elastic electron scattering studies as very few surprises emerged. Also, the unfortunate fact was soon discovered that large values of polarisation were only obtained in the scattering minima. This is because the polarisation is caused by an angular displacement of the scattered intensity for electrons of different spin directions, but the maxima are broad and thus insensitive to the displacement. In other words, electron scattering has only yielded very small currents of polarised electrons, so interest in ways of producing polarised electrons rapidly switched to other fields.

The electron scattering maxima and minima are found at electron energies ranging from a few electron volts up to many hundred, especially in the heavier atoms. Quite deep minima in the scattering intensities are observed at scattering angles ranging from beyond the forward peak to well into the backward scattering region.

The new electron diffraction was discovered by Geiger and Morón-León at higher electron energies and at scattering angles of less than 0.5° . It occurs not by diffraction on the atom itself but from a kind of shadow around the atom produced by other scattered electrons. This diffraction had not been predicted theoretically, although the reason for its occurrence is easily understood.

In general, the intensity of electrons which are scattered elastically at any scattering angle is larger than the intensity of electrons which have lost some of their energy, for example by exciting or ionising the atom. These inelastically scattered electrons, however, dominate the forward scattering, especially at high energies, where their intensity is typically 1000 times greater than the elastic. So if only the



Shadow diffraction in neon at electron energies of 15, 20 and 25 keV. For clarity the three curves have been displaced and the orders of the diffraction minima have been numbered.

THE statement on page 4 of the *News and Views* article prepared by Leffert and Koch, entitled 'Ionic Landmarks Along the Mitogenic Route' (*Nature*, **279**, 104; 1979) is incorrect. Proteolysis does not convert myelin basic protein into a mitogenic peptide whose sequence is Thr-Pro-Pro-Ser-Gln-Gly-Lys. What I stated at the conference was that from our data the brain FGF activity resides within region 91-117 of the basic protein. However because of the effects of other portions of the molecule on mitogenicity one would not expect that this region itself would be mitogenic.

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elastically scattered electrons are detected, it appears as if the atoms were surrounded by large opaque fuzzy-edged disks, or shadows, since almost no elastically scattered electrons can appear at small scattering angles. It is these disks which are responsible for the diffraction effects.

It is clear by comparison with light diffraction that diffraction effects become easier to observe when the diffracting object is not very large compared with the wavelength of the diffracted beam. So it is not surprising that shadow diffraction has remained undetected for so long. The experimental techniques necessary for its observation are more difficult than for large angle scattering, and there was no theoretical prediction.

The apparatus used by Geiger and Morón-León is similar to an electron microscope in which the specimen is replaced by the target gas at a pressure of about 0.1 Torr. A diminished electron optical image, 8 μm in diameter, of the cathode is formed in a filter lens. This is so arranged that all electrons which have lost energy by any inelastic collision with the target atoms see a potential hill which they cannot surmount, and so fall back; that is, they are reflected by the filter lens. The remaining elastically scattered electrons are imaged by the filter lens onto a photographic plate.

Measurements were made in helium, neon and argon. Typical results obtained with neon are shown in the figure, from which it is clear that the diffraction effects are more pronounced at lower energies. The well-known Born approximation, which is flat over this narrow angular range, is used for normalisation purposes. The orders of the diffraction minima are numbered. Geiger and Morón-León assume that the radius R of the disk is equivalent to a 0th order diffraction maximum of angular full width at half height $\theta = \Delta E/E$ where ΔE is the energy lost by the inelastically scattered electrons of primary energy E . This gives an energy dependent radius R of the disk given by $R = 2/k\theta$, where k is the wavenumber of the incident electrons. In the case of the 15 keV electrons scattered from neon, if it is assumed that the main energy loss, ΔE , is due to excitation of the resonance line at 16.9 eV, the radius of the scattering disk is 30 Å, or some 23 times larger than the kinetic theory atomic radius. This agrees exactly with the value of the radius which can be obtained by substituting the measured minima shown in the figure in the Fraunhofer diffraction equation. Good agreement is also obtained in all other cases, where the diffraction angles are not so accurately measurable. So the remarkably simple model of an opaque shadow disk without a fuzzy edge gives an

excellent explanation of the observations.

Clearly shadow diffraction now awaits a more detailed theoretical treatment, preferably including electron spin effects. Rapidly changing intensities are an indication that spin polarisation effects are to be expected. For example, in addition to the spin polarisation measurements

discussed above, electrons elastically scattered in the neon resonances were shown to be polarised by Franzen and Gupta in 1965. Since spin-up and spin-down electrons could see a different-sized shadow, shadow diffraction may well produce polarised electrons where the intensities are by no means small. □

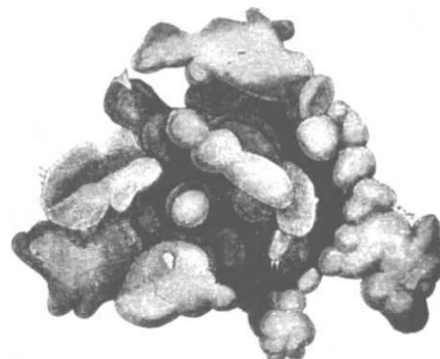


100 years ago

THE eminent zoologist, Dr. Karl Moebius, of Kiel, has recently published a treatise, "Der Bau des *Eozoon canadense* nach eigenen Untersuchungen verglichen mit der Bau der Foraminiferen" ("The Structure of *Eozoon canadense*, according to my own Investigations, compared with the Structure of Foraminifera"), which first appeared in the "Palaeontographica" (vol. xxv.), and was afterwards republished separately. Prof. Moebius inclines entirely towards the view of King and Rowney (*Proc. Roy. Irish Acad.* ser. 1, x. and ser. 2, i.) disputing the organic character of *Eozoon*. . .

According to Dr. Moebius *Eozoon canadense* consists principally of alternate layers of yellowish green serpentine and whitish limestone. . .

"If the *Eozoon* pieces from the Laurentian or 'Urgneiss' formation



were really remains of an undoubted foraminifera species, then we should possess in them *certain* proofs that even during the formation of the most ancient strata of the earth's crust living beings occurred, and that the first organisms belonged to the lowest animals, by which biology and geology would have gained two highly important facts. Yet by the scientifically justified elimination of *Eozoon* from the domain of organic beings it is not proved that during the Laurentian period no living beings existed. Perhaps the graphite of the Urgneiss formation has its origin in organic beings."

from *Nature* 20, 17 July, 272, 24 July, 300; 1879.



review article

Protein methylation in behavioural control mechanisms and in signal transduction

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In both prokaryotes and eukaryotes methyl groups can be added to and removed from the carboxyl groups of proteins. Recent work has revealed that these reactions have a role in several behavioural phenomena. The nature of this role has been uncovered in one case—that of bacterial chemotaxis.

AMONG the problems addressed by biologists, one of the most intriguing is that of defining the mechanisms that underlie behaviour at the cellular level. This includes the transduction of one signal into another. For example, sensory stimuli interact with sensory receptors to bring about an alteration in cellular output, neurotransmitters combine with synaptic receptors to change ion fluxes, and hormones affect cell surface receptors to produce responses in target cells. In the past few years it has become apparent that reversible covalent modifications of macromolecules play an important role in some of these mechanisms. The phosphorylation and dephosphorylation of proteins are perhaps the best characterised examples¹⁻⁴. Recently, another class of covalent modification, methylation of protein-carboxyl groups, has been linked to the control of behaviour and to signal transduction in both prokaryotes and eukaryotes. In this article we will summarise the evidence for the involvement of protein methylation in bacterial chemotaxis, a simple behavioural response in which the role of this modification has been characterised in some detail. We will also provide a brief discussion of the evidence linking protein methylation to behaviour in eukaryotes.

The response of bacteria to chemical stimuli

Motile bacteria such as *Escherichia coli* and *Salmonella typhimurium* are attracted by certain chemicals and repelled by others, a phenomenon known as chemotaxis⁵⁻¹⁵. In nature the cells respond to spatial gradients of these chemicals by monitoring concentration as a function of time and reacting to the changes experienced as they swim from one place to another. The bacteria, then, are actually sensitive to temporal changes in chemical concentration¹⁶⁻¹⁸. In the laboratory it is convenient to provide temporal stimuli directly, by the sudden addition or removal of a chemical^{16,17}.

Before stimulation, the cells show a pattern of motility characterised by periods of smooth swimming interrupted at random intervals by brief periods of chaotic tumbling^{16,19}. Smooth swimming is produced by anticlockwise rotation of the flagella²⁰, which act as propellers to drive the cells through the surrounding medium²¹⁻²³. Tumbling results when the direction of rotation is reversed^{20,24}.

When an attractant is added (Fig. 1a), the cells respond with a suppression of tumbling¹⁶. After a lag period, however, the frequency of tumbling returns to the pre-stimulus level^{16,25,26}; the cells have in effect become insensitive to the continued presence of the attractant. The initiation of the behavioural response is referred to as sensory excitation and the subsequent loss of sensitivity as sensory adaptation. The adaptation process begins as soon as the attractant is added and proceeds until it reaches completion and terminates the response²⁶. The time required for this is a function of the strength of the stimulus (defined by the magnitude of the increase in concentration) and can be many minutes following a large concentration change^{16,25,26}.

When the attractant is removed, sensitivity is rapidly regained²⁶, that is, the bacteria can respond again if the attractant is added back. The restoration of sensitivity can be thought of as the inverse of adaptation and is referred to as de-adaptation. A curious asymmetry should be pointed out: the de-adaptation process is very much faster than the corresponding

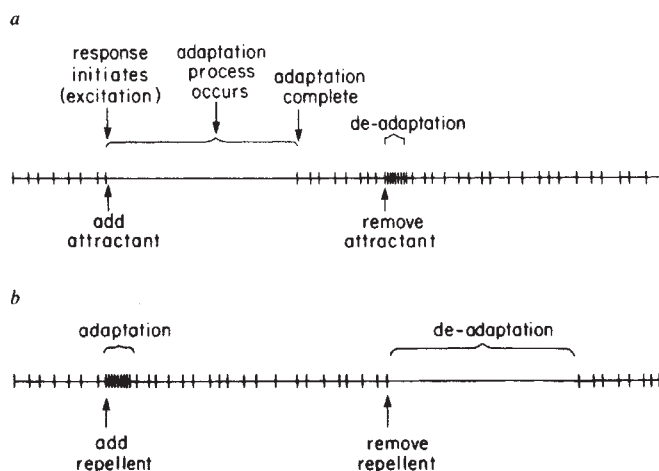


Fig. 1 Schematic representation of temporal responses. Each vertical line represents a tumble. The absence of vertical lines that follows addition of attractant or removal of repellent represents suppression of tumbling, while the increased frequency of lines that follows removal of attractant or addition of repellent represents continuous tumbling. *a*, Effect of attractants; *b*, effect of repellents.

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adaptation process, requiring no more than a few seconds even for a large decrease in attractant concentration^{16,26}. This asymmetry is indicated in Fig. 1a. Note that de-adaptation is accompanied by a brief period of enhanced tumbling^{16,26}.

Repellents have behavioural effects which by several criteria are opposite to those described for attractants. First, addition of a repellent (like removal of an attractant) results in a short period of increased tumbling, whereas removal of repellent (like addition of an attractant) causes a longer period of suppressed tumbling¹⁷ (Fig. 1b). Second, if an attractant and a repellent are added simultaneously the two tend to cancel each other's effects^{17,27}. These results suggest that, in terms of the underlying biochemical mechanisms, there is an analogy between the process which follows addition of repellent and that which follows removal of attractant and similarly between removal of repellent and addition of attractant.

The mechanisms that underly these behavioural responses have been analysed by using mutants that are defective in various aspects of chemotaxis. The mutants fall into several classes which can be used to define a pathway of information flow, as shown in Fig. 2. The first class, called specifically non-chemotactic, suffer from lesions in one or another of the different kinds of chemoreceptors²⁸ that detect specific compounds. These mutants respond normally to most chemicals and lack responses only to those chemicals detected by the receptor that has been affected²⁸⁻³⁴. The second class contains mutants that have lost responsiveness to the chemicals detected by several different kinds of receptor, but that retain responsiveness to compounds detected by the remaining receptors. This class of mutants, known as multiply nonchemotactic mutants, contains three complementation groups, called *tsr*, *tar* and *trg*, which define three major branches of the pathway³⁵⁻³⁹ (see Fig. 2). The receptors that feed information through the branch defined by the *tsr* gene are called type I receptors, and those feeding through *tar* and *trg* are called type II and type III, respectively. Ultimately, information from all the receptors is integrated and used to control the direction of rotation of the flagella and consequently the frequency of tumbling, as described above. It is in this final, integrated pathway that a third class of mutants is found. These are the generally non-chemotactic (*che*) mutants; they fall into eight complementation groups^{15,28,40,41}. A last class, the non-motile mutants, has lost the ability to synthesise or rotate the flagella^{15,42} (Fig. 2).

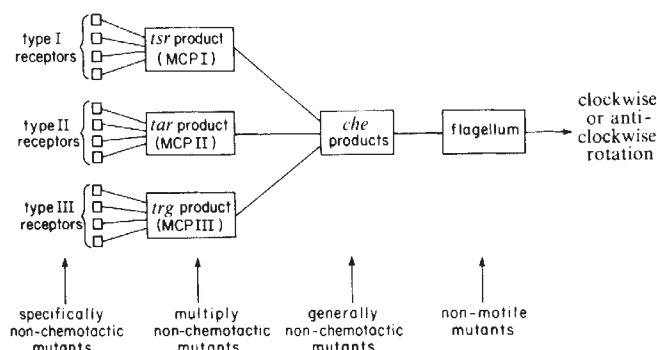


Fig. 2 Information flow during bacterial chemotaxis. The lines represent the pathways of information flow; each of the empty boxes represents a different kind of chemoreceptor. The arrows at the bottom indicate where this flow of information has been interrupted in the various mutants.

Information flow through methylated proteins

Several years ago it was discovered that a protein methylation reaction has an essential role in bacterial chemotaxis⁴³. Two criteria were used to demonstrate this. First, the extent of methylation of several cytoplasmic membrane proteins^{35,36,39} was shown to be altered by chemicals that elicit a chemotactic response⁴³. Second, the methylation reaction failed to occur in

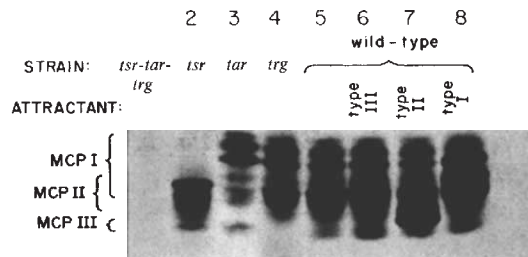


Fig. 3 Methylation patterns from a chemotactically wild-type strain and from *tsr* (lacking MCP I), *tar* (lacking MCP II) and *trg* (lacking MCP III) mutants. Bacteria were incubated with [Me^3H]methionine in the presence of a protein synthesis inhibitor, as described previously³³. The attractants used, D-ribose (5 mM), DL- α -methylaspartate (5 mM) and α -aminoisobutyrate (50 mM), are type III, type II and type I stimuli, respectively. Equal amounts of protein were layered on a 7% SDS polyacrylamide gel. The fluorographic image shows the region of the gel where proteins of molecular weights 56,000–65,000 are found. The brackets indicate the bands which compose MCP I, MCP II and MCP III. The lower two bands of MCP I and the upper two bands of MCP II overlap³⁵. Strains used were AW405, AW518, AW539, AW701 and AW660 for the wild type, *tsr*, *tar*, *trg* and *tsr-tar-trg* mutants, respectively.

some generally non-chemotactic mutants and, when such a mutant was reverted for chemotaxis, methylation simultaneously reappeared⁴³. The proteins were therefore called the methyl-accepting chemotaxis proteins (MCPs).

Where do the MCPs function in the pathway of information flow described above? Three different MCPs have been defined by the three complementation groups of the multiply non-chemotactic mutants^{35,36,39}. This is documented in Fig. 3, which shows the methylation patterns obtained by incubating bacteria with [Me^3H]methionine (the source of the methyl group). As shown in Fig. 3 (lanes 1–5), the *tsr* mutant lacks a set of methylated bands collectively called MCP I (refs 35, 36), the *tar* mutant a set of bands called MCP II (refs 35, 36) (note that there is some overlap of MCP I and MCP II), and the *trg* mutant a set of bands called MCP III (ref. 39); the triple mutant lacks all these methylated bands³⁹. Evidence suggests that *tsr* and *tar* actually code for the MCP I and II polypeptides, respectively^{36,44}, and by analogy it is thought that *trg* may code for the MCP III polypeptides³⁹. Why each of these genes gives rise to more than one polypeptide is not clear, although there is some indication that this results from post-translational modification of the gene products⁴⁵.

Information from each of the three different types of receptor (as defined in Fig. 2) causes a change in the level of methylation of the appropriate MCP. Apparently, the cells maintain a basal level of methylation in the absence of external stimuli. If a type I, II or III attractant is added there is a large increase in the level of methylation of the appropriate MCP with little or no increase in the methylation of the other two MCPs^{35,36,39,46}. This is shown in Fig. 3, lanes 5–8. As illustrated in Fig. 4a, the basal level of methylation of an MCP (defined by the plateau of radioactivity incorporated by cells from labelled methionine in the absence of added stimuli^{43,46}) is rapidly increased in response to an attractant until a new plateau is reached. The methylation will remain at this new level as long as the attractant is present, and returns to the basal level only when the attractant is removed⁴⁶. Addition of a repellent, on the other hand, causes a decrease in methylation⁴⁶ (Fig. 4b), and removal of repellent causes re-methylation (M.S.S. and M.F.G., unpublished); these results might be expected as repellents and attractants have opposite effects on behaviour.

The chemical reactions which are used to regulate the level of methylation of the MCPs are shown in Fig. 5. Methyl groups are transferred from S-adenosylmethionine to the MCPs⁴⁷ by esterification of the γ -carboxyl group of one or more glutamic acid residues^{48,49}. Methyl groups are removed by a reaction in which the final product is methanol^{50,51}. Both reactions have been demonstrated in *in vitro* systems^{47,50}. A methyl transferase has been partially purified and found to be a soluble protein with

a molecular weight of about 41,000 (ref. 47). An esterase has also been identified; it is a soluble protein and has a molecular weight of about 100,000 (ref. 50).

Involvement of methylation in sensory adaptation

What role do these changes in methylation play in the chemotactic response? This question can be investigated by observing the behaviour of cells in which the methylation reaction has been blocked. The discovery that *cheX* mutants of *E. coli* have the acceptor proteins present in the membrane but lack the ability to methylate them^{35,36,43,47} provided an opportunity for this type of experiment. A number of independent *cheX* mutants have been examined: each initiated the appropriate behavioural responses when subjected to attractant and repellent stimuli, but in each the responses were abnormally prolonged^{52,53}. For example, wild-type cells typically exhibit a 5-min period of smooth swimming following the addition of the attractant α -aminoisobutyrate (50 mM final concentration) but a *cheX* mutant failed to adapt to this stimulus even after 36 h of exposure⁵³. It seems, then, that the process of sensory adaptation is defective in these mutants.

Methylation has also been blocked by starving methionine auxotrophs of methionine and consequently depleting them of

the methyl donor for the reaction. Cells partially starved of methionine respond to the addition of attractants but do so for abnormally long periods^{54,55}. The severity of this adaptation defect increases as the bacteria become further depleted of methionine⁵⁵ until finally adaptation fails entirely⁵⁶. Thus, two independent methods of blocking methylation lead to the same conclusion: methylation is involved in the process of sensory adaptation.

The changes in methylation level following addition or removal of stimuli (see Fig. 4) could serve as the biochemical mechanisms which regulate the processes of adaptation and de-adaptation^{46,56}. This hypothesis may be summarised as follows: (1) the level of methylation of the MCPs observed before a stimulus is added corresponds to a state in which the cell is fully sensitive to the stimulus; (2) the increase in methylation level that follows the addition of an attractant constitutes the process of adaptation to the attractant; (3) the final level of methylation corresponds to the state of complete adaptation to the attractant, and maintenance of the level corresponds to the maintenance of that state; (4) demethylation, which results from removal of the attractant, constitutes the process of de-adaptation whereby sensitivity is re-established; (5) for repellents, as suggested by the opposite nature of the behavioural responses, the adaptation and de-adaptation processes are reversed relative to attractants, so that the decrease in methylation that follows addition of repellent represents the process of adaptation, whereas the increase in methylation that follows its removal corresponds to the process of de-adaptation.

There is considerable evidence to support this proposal. First, the methylation and demethylation reactions show the same asymmetry as the adaptation and de-adaptation processes^{16,17,26,46}. Specifically, methylation, like the process of adaptation which follows addition of an attractant, is slow, while demethylation, like the process of de-adaptation which follows removal of an attractant, is fast (Figs 1a, 4a). This analogy holds true in reverse for repellents. Second, if the methylation reaction actually controls the adaptation process, the cells should respond to an attractant only while the level of methylation is increasing. Data indicate that the time required for the increase in methylation is very similar to that required for adaption⁴⁶. In fact, when two stimuli are compared it is found that the ratio of the length of the responses to these two stimuli is equal to the ratio of the half-times of the changes in methylation induced by the stimuli⁴⁶. Third, there is a striking correlation between the methionine dependency of the behavioural response and the methionine dependency of methylation^{46,56}. Adaptation to attractants is methionine dependent, but once a cell has adapted to an attractant it will remain adapted even if methionine is subsequently removed; furthermore, de-adaptation can occur in the absence of methionine⁵⁶. Correspondingly, it has been demonstrated that methylation requires the presence of methionine, but that both maintenance of an attractant-induced level of methylation and demethylation when the attractant is removed do not⁴⁶. Taken together, these observations support the hypothesis that the state of methylation of the MCPs regulates the state of adaptation of the cell.

The control of the level of methylation

As seen in the preceding sections, the methylation and demethylation of the MCPs are carefully regulated. A study of the generally non-chemotactic mutants suggests that the products of some of the *che* genes play a part in the control of these processes. The *che* mutants can be divided into two groups based on their methylation phenotypes.

cheX and *cheB* mutants of *E. coli* (*cheR* and *cheX* of *S. typhimurium*) incorporate virtually no radioactive methyl groups into the MCPs^{35,36,43,47}. Present evidence indicates that the product of the *cheX* gene is the methyl transferase⁴⁷, and the product of the *cheB* gene is the methyl esterase^{50,57}. (Apparently, *cheB* mutants fail to incorporate label into the MCP polypeptides because they are always fully methylated⁵⁷

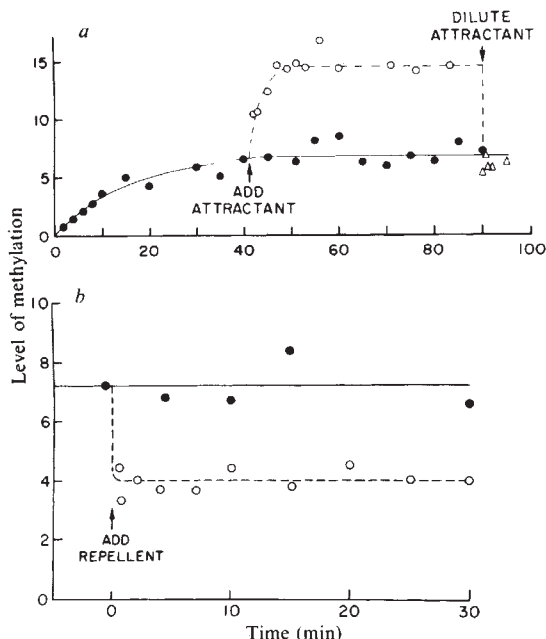


Fig. 4 Effect of chemical stimuli on the methylation of MCP (from ref. 46). Samples were electrophoresed on a 12% SDS polyacrylamide gel. A fluorographic image of the gel was obtained and the MCP region in the developed film scanned with a densitometer. The 'level of methylation' of each sample is the area under the densitometric curve divided by the amount of protein applied to the gel, and is expressed in arbitrary units. The data were obtained by scanning the entire MCP region and thus represent the behaviour of aggregate MCP and not that of any one band. *a*, [^3H]methionine was added at time zero in the presence of a protein synthesis inhibitor. After 40 min incubation the culture was divided in two. One group of cells (○) was stimulated by the addition of the type I attractant α -aminoisobutyrate (50 mM final concentration), the other group of cells (●) received no addition. The concentration of α -aminoisobutyrate was subsequently lowered by diluting the stimulated cells 100-fold into medium containing no α -aminoisobutyrate (Δ); the first point was obtained 15 s after this dilution. (A 100-fold dilution of stimulated cells into medium containing α -aminoisobutyrate had no effect on the level of methylation.) Similar data have been obtained for type II and type III attractants. *b*, Cells were incubated with [^3H]methionine for 30 min and the culture divided in half. One part received no addition (●) and a mixture of type I repellents was added to the second (○). Zero time refers to the time at which the stimulus was added. The first sample was obtained 20 s after stimulation. When the repellent was removed (at 2 min) by filtration and the cells resuspended in medium containing [^3H]methionine, the level of methylation returned to its initial value 20 min after filtration, the earliest time studied (data not shown, M.S.S. and M.F.G., unpublished).

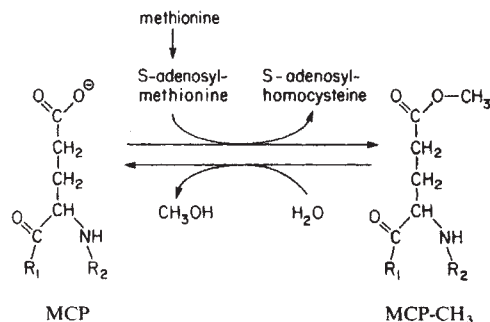


Fig. 5 Protein modification reactions discussed in this article.

and cannot demethylate to provide sites for the incorporation of labelled methyl groups.) Interestingly, lesions in either of these genes affect the methylation of all three MCPs^{35,36,43,47,50,57}, so the methylating and demethylating enzymes are probably not specific for any single MCP. From biochemical studies⁸⁴ it seems that the specificity required to permit a particular stimulus to affect the methylation of only MCP I or MCP II or MCP III results from physical contact between a chemoreceptor and one of the three MCPs, an interaction that alters the ability of that MCP to be methylated or demethylated.

Mutations in the other *che* genes produce strains that do incorporate radioactive methyl groups, although in some cases the amount and/or the distribution of label incorporated into the various MCPs is altered (refs 35, 36, 43, 47 and M.S.S. and M.F.G., unpublished). However, no interpretation of these defects has been formulated, and the functions of the products of these genes remain unknown.

Sensory excitation in bacterial chemotaxis

In the above discussion the chemotactic response has been separated into two processes, called excitation and adaptation. The validity of this separation is demonstrated best by the *cheX* mutants, which become excited in response to stimuli but fail to adapt. Excitation, like adaptation, seems to involve the MCPs^{35,36}, as mutations in *tsr*, *tar* and *trg* (the genes that apparently code for MCP I, II, and III) produce strains that are defective in excitation of type I, II and III stimuli, respectively^{35,36,39,58}, and a triple mutant carrying all three lesions is unable to produce an excitatory response to any stimulus tested (C. B. Ball and H. Kondoh, unpublished). However, unlike adaptation, excitation does not involve methylation of the MCPs, as *cheX* mutants, which have the MCPs but cannot methylate them, can still give an excitatory response^{52,53}. The molecular details of the excitatory process remain to be elucidated.

A working model for the role of methylation in bacterial chemotaxis

Regulation of the chemotactic response is ultimately manifested as control over whether the flagella rotate in the anticlockwise or clockwise direction and hence whether the cell swims smoothly or tumbles. Some parameter, which we call the rotation regulator, must determine the sense of this rotation. (In previous work this parameter has been called the response regulator or the tumble regulator^{9,12,14}.)

In our model there are two processes that involve the MCPs: an excitatory process, whose activity changes rapidly when a stimulus is presented, and an adaptational process, whose activity changes slowly (Fig. 6a). It is the ratio of these two activities that determines the level of rotation regulator. When the system is at rest (that is, when the cell is not responding to any stimulus) the two processes are balanced so that the rotation regulator is present at a level that allows both smooth swimming and tumbling (see Fig. 6). If an attractant is added, the receptors immediately produce a signal that results in a rapid increase in the activity of the excitatory process, and the level of the rotation regulator rises. The flagella rotate exclusively anticlockwise and tumbling is suppressed (that is sensory excitation occurs). At the

same time, the receptor signal also causes a slow increase in the activity of the adaptation process, namely, an increase in the level of methylation of the appropriate MCP. This tends to counteract the effect of the excitational process, and the level of the rotation regulator begins to return to the resting level. Ultimately, when the new plateau level of methylation is reached, the two processes are again in balance, and the level of the rotation regulator has returned to a point that allows both smooth swimming and tumbling (Fig. 6b, c). The cell has adapted, and the response is terminated. When the attractant is removed, both the excitatory process and the level of methylation fall to their original value. However, the removal of methyl groups lags slightly behind, and there is a brief decrease in the level of the rotation regulator, resulting in a brief tumbling response. Once this is completed the cell has de-adapted and is ready to respond again. (According to this model the response to addition of a repellent would be exactly analogous to the response to removal of an attractant, and removal of repellent would be like addition of attractant.) A molecular interpretation is presented in Fig. 7.

Our model is representative of a class of models which use two competing processes to initiate and terminate behavioural responses⁵⁹⁻⁶¹. Such two-process models have been successfully applied previously to explain the properties of the chemotactic response in bacteria^{16,26}. Although our version accounts for most of the phenomena of chemotaxis, including the relationship of methylation to the response, there are several observations which do not fit well into the framework as presented.

The model predicts that *cheX* strains, which do not have the complete apparatus with which to methylate their MCPs⁴⁷, should not tumble at all (since the activity of the excitational process should always exceed that of the adaptation process) whereas *cheB* strains, whose MCPs are permanently methylated^{50,57}, should tumble continuously (since the activity of the adaptation process should always exceed that of the excitational process). Indeed, this is true in most conditions^{14,15}. However, *cheX* mutants can be made to tumble by the addition of a repellent^{47,52,53} and *cheB* mutants can be made to swim smoothly by addition of an attractant¹⁴; thus, additional studies of these mutants and/or refinements of the model are required.

Several important questions pertaining to the methylation and demethylation reactions remain unanswered. First, the identity of the rotation regulator has not been established, although evidence has been presented that in *Bacillus subtilis* it may be intracellular calcium ions⁶². Second it remains to be seen how the MCPs function to control the level of the rotation regulator. For example, they could be enzymes that synthesise the rotation regulator, or they could be ion gates that control the

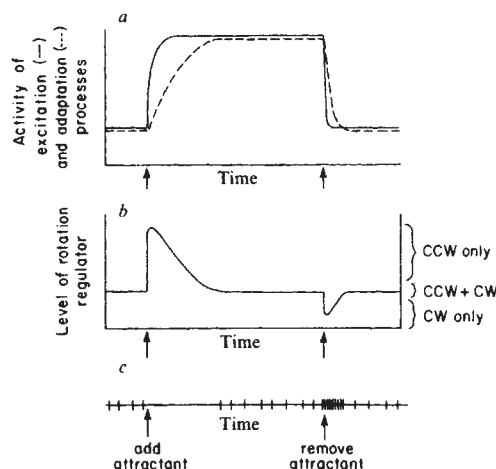


Fig. 6 Model for the role of methylation in bacterial chemotaxis. CCW stands for anticlockwise rotation of the flagella, which corresponds to smooth swimming, and CW stands for clockwise rotation, which corresponds to tumbling. Figure 6c is repeated from Fig. 1a. See text for explanation. (Increase in level of rotation regulator producing anticlockwise rotation is an arbitrary choice; it could just as well be a decrease.)

level of an ion which serves as the regulator. Third, we do not know the details of how the binding of stimulatory chemicals to chemoreceptors influences the methylation of the MCPs.

Despite the work that remains to be done, the identification and characterisation of the methylation and demethylation reactions represent a significant step forward in the effort to understand how bacteria transduce sensory signals into a behavioural response.

Carboxyl methylation in eukaryotic systems

The existence of protein-carboxyl methylation in eukaryotes was known before the discovery of the reaction in bacteria⁶³⁻⁷¹. However, its function in eukaryotic cells is only now beginning to be understood. This reaction seems to regulate several processes, some behavioural in nature. The one most closely related to the bacterial system described above is leukocyte chemotaxis.

A number of different types of white blood cells use chemotaxis in the initial phase of their response to infectious or inflammatory agents⁷². Many natural and synthetic substances act as attractants for these cells⁷³. During the past two years several groups have investigated the possibility that a carboxyl methylation reaction might be involved in this phenomenon^{74,75}. Measurement of the methanol released when carboxyl-methyl esters are hydrolysed at high pH⁷⁴ has shown that the addition of an attractant to leukocytes causes a perturbation of the level of carboxyl methylation⁷⁴, and that both the behavioural response and the change in carboxyl methylation can be prevented by the prior addition of an attractant antagonist⁷⁶. Furthermore, it has been found that incubation of leukocytes with compounds that inhibit methylation reactions in general^{75,77,78} blocks the behavioural response^{75,79}. Thus methylation seems to be involved in the sensory response of leukocytes.

It is interesting that methyl-acceptor proteins and a methylating activity are also found in high concentrations in mammalian testis, spermatazoa and spermatids^{66,71,80}. It is possible that here, too, there is involvement of protein methylation in a chemotactic response. Although there is at present no evidence for or against the idea, it may be that mammalian spermatazoa use chemotaxis as an aid to fertilisation; certainly, this occurs in simpler animals⁸¹.

Protein-carboxyl methylation also plays a part in neural and endocrine functions, as indicated by the following results. (1) Protein-carboxyl methylase is found in most mammalian organs but most abundantly in nervous tissue and endocrine glands, for example brain and pituitary^{66,70,71}. Methyl-acceptor proteins are also widely distributed, the pituitary gland being the richest source^{70,71}. (2) The methylating enzyme is apparently transported down axons and occurs in synaptosomal preparations⁷¹. A function at the synapse is indicated by the fact that synaptosomes from the hypothalamus undergo changes in the level of methylation following depolarisation⁸². (3) Stimulation of the adrenal medulla, which results in the secretion of catecholamines, produces an increase in methylating activity^{76,83}. When methylation is blocked by an inhibitor, secretion does not occur⁷⁶. (4) In the parotid gland β -adrenergic stimulation, which releases amylase, is accompanied by an increase in the level of methylation of methyl acceptor proteins⁸⁰.

Taken together, these results suggest that some of the functions of carboxyl methylation in eukaryotes, as in prokaryotes, concern signal transduction and the control of behaviour at the cellular level.

Conclusion

The methylation and demethylation of protein-carboxyl groups are reactions which may play important parts in the regulation of cellular activities. Protein-carboxyl methylation has already been implicated in several behavioural control mechanisms in a variety of prokaryotes and eukaryotes. That the widespread occurrence of this reaction has only recently been recognised is due at least in part to the lability of carboxyl esters which hinders

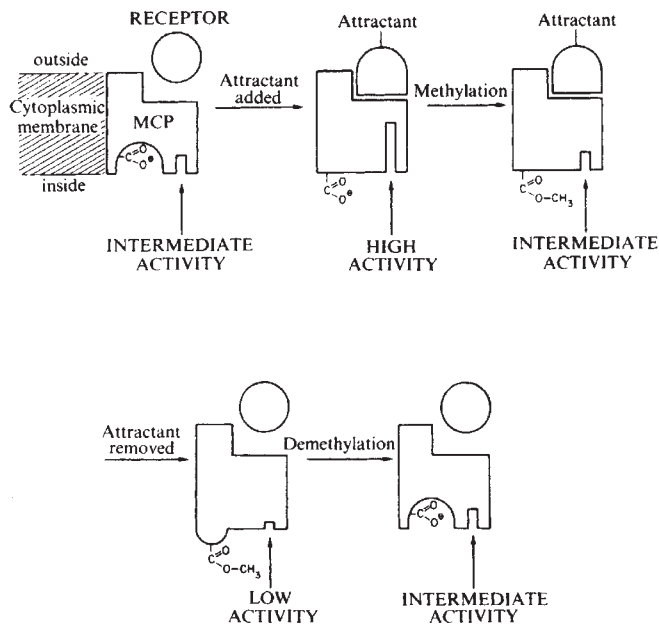


Fig. 7 Speculative molecular model for effect of attractant on the activity of MCP. This activity (ability to generate rotation regulator) is indicated in the figure at various points in the response. In the unstimulated state (top left) MCP has intermediate activity leading to an intermediate level of rotation regulator, and thus both anticlockwise and clockwise rotation can occur (see Fig. 6). When the attractant is added (top centre), it binds to the chemoreceptor and brings about a physical interaction between the receptor and the appropriate MCP. This has two effects: (1) via the excitation process, there is a rapid increase in activity and thus a rise in the level of rotation regulator, so that only anticlockwise rotation occurs; and (2) carboxyl groups become exposed and available for methylation. Slowly methylation then takes place (top right) and causes the activity to return to the intermediate level; thus adaptation occurs. Removal of attractant (bottom left) decreases the activity of MCP; this leads to a decrease in the level of rotation regulator and thereby produces clockwise rotation. Demethylation (bottom right) restores the activity to the unstimulated level. As discussed in the text, neither the nature of the rotation regulator nor the means of its generation by MCP is known. The model shows several features which have been experimentally determined. First, MCP is believed to span the cytoplasmic membrane: interaction of chemical stimuli with chemoreceptors must take place on the outside of the cytoplasmic membrane, and at least some of the receptors are found in the periplasm (the space between the cytoplasmic membrane and the cell wall)⁸; methylation and demethylation must take place on the inner surface of the cytoplasmic membrane, since the enzymes that catalyse these reactions are cytoplasmic^{47,50}. Second, upon interaction with ligands, conformational changes in chemoreceptors have been described^{85,86}; these observations have been used previously to postulate a ligand-induced conformational change as an obligatory first step prior to the interaction of receptor with subsequent components of the chemotaxis machinery⁸⁷. Third, chemoreceptors appear to interact with MCP directly⁸⁴.

their identification by usual analytical techniques. With improved methodology protein-carboxyl methylation may be found as ubiquitously as the better known protein phosphorylation reactions.

Bacteria such as *E. coli* and *S. typhimurium* offer a favourable system for the study of the relationship of protein methylation to behaviour, because these organisms are relatively simple and well characterised both biochemically and genetically. From studies of sensory transduction in bacteria a set of facts and concepts may emerge that is generally applicable to our understanding of the phenomena that underlie behaviour and signal transduction in higher organisms, as evolution seems to have conserved at least some of the biochemical mechanisms involved.

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1. Hubbell, W. L. & Bownds, M. D. A. *Rev. Neurosci.* **2**, 17-34 (1979).
2. Greengard, P. *Nature* **260**, 101-108 (1976).
3. Nathanson, J. A. *Physiol. Rev.* **57**, 157-256 (1977).
4. Greengard, P. *Science* **199**, 146-152 (1978).
5. Engelmann, T. W. *Pflügers Arch. ges. Physiol.* **25**, 285-292 (1881).
6. Pfeffer, W. *Unters. bot. Inst. Tübingen* **1**, 363-482 (1884).
7. Adler, J. *Science* **153**, 708-716 (1966).
8. Adler, J. A. *Rev. Biochem.* **44**, 341-356 (1975).
9. Berg, H. C. A. *Rev. Biophys. Bioengng.* **4**, 119-136 (1975).
10. Goy, M. F. & Springer, M. S. in *Taxis and Behavior, Receptors and Recognition*, ser. B, Vol. 5 (ed. Hazelbauer, G. L.) 1-34 (Chapman and Hall, London, 1978).
11. Hazelbauer, G. L. & Parkinson, J. S. in *Microbial Interactions, Receptors and Recognition*, ser. B, Vol. 3. (ed. Reissig, J. L.) 61-98 (Chapman and Hall, London, 1977).
12. Koshland, D. E. *Jr Science* **196**, 1055-1063 (1977).
13. Macnab, R. M. *CCR Critical Rev. Biochem.* **5**, 291-341 (1978).
14. Parkinson, J. S. A. *Rev. Genet.* **11**, 397-414 (1977).
15. Silverman, M. & Simon, M. I. A. *Rev. Microbiol.* **31**, 397-419 (1977).
16. Macnab, R. M. & Koshland, D. E. *Jr Proc. natn. Acad. Sci. U.S.A.* **69**, 2509-2512 (1972).
17. Tsang, N., Macnab, R. & Koshland, D. E. *Jr Science* **181**, 60-63 (1973).
18. Brown, D. A. & Berg, H. C. *Proc. natn. Acad. Sci. U.S.A.* **71**, 1388-1392 (1974).
19. Berg, H. C. & Brown, D. A. *Nature* **239**, 500-504 (1972).
20. Larsen, S. H., Reader, R. W., Kort, E. N., Tso, W. W. & Adler, J. *Nature* **249**, 74-77 (1974).
21. Berg, H. C. & Anderson, R. A. *Nature* **245**, 380-382 (1973).
22. Silverman, M. & Simon, M. *Nature* **249**, 73-74 (1974).
23. Berg, H. C. *Nature* **249**, 77-79 (1974).
24. Khan, S., Macnab, M., DeFranco, A. L. & Koshland, D. E. *Jr Proc. natn. Acad. Sci. U.S.A.* **75**, 4150-4154 (1978).
25. Spudich, J. L. & Koshland, D. E. *Jr Proc. natn. Acad. Sci. U.S.A.* **72**, 710-713 (1975).
26. Berg, H. C. & Tedesco, P. M. *Proc. natn. Acad. Sci. U.S.A.* **72**, 3235-3239 (1975).
27. Adler, J. & Tso, W.-W. *Science* **184**, 1292-1294 (1974).
28. Adler, J. *Science* **166**, 1588-1597 (1969).
29. Hazelbauer, G. L. & Adler, J. *Nature new Biol.* **230**, 101-104 (1971).
30. Aksamit, R. & Koshland, D. E. *Jr Biochem. biophys. Res. Commun.* **48**, 1348-1353 (1972).
31. Adler, J. & Epstein, W. *Proc. natn. Acad. Sci. U.S.A.* **71**, 2895-2899 (1974).
32. Hazelbauer, G. L. *J. Bact.* **122**, 206-214 (1975).
33. Lengeler, J. *J. Bact.* **124**, 39-47 (1975).
34. Galloway, D. R. & Furlong, C. E. *Archs Biochem. Biophys.* **184**, 496-504 (1977).
35. Springer, M. S., Goy, M. F. & Adler, J. *Proc. natn. Acad. Sci. U.S.A.* **74**, 3312-3316 (1977).
36. Silverman, M. & Simon, M. *Proc. natn. Acad. Sci. U.S.A.* **74**, 3317-3321 (1977).
37. Ordal, G. W. & Adler, J. *J. Bact.* **117**, 517-526 (1974).
38. Reader, R. W., Tso, W.-W., Springer, M. S., Goy, M. F. & Adler, J. *J. gen. Microbiol.* **111**, 363-374 (1979).
39. Kondoh, H., Ball, C. B. & Adler, J. *Proc. natn. Acad. Sci. U.S.A.* **76**, 260-264 (1979).
40. Armstrong, J. B., Adler, J. & Dahl, M. M. *J. Bact.* **93**, 390-398 (1967).
41. Parkinson, J. S. *J. Bact.* **135**, 45-53 (1978).
42. Iino, T. A. *Rev. Genet.* **11**, 161-182 (1977).
43. Kort, E. N., Goy, M. F., Larsen, S. H. & Adler, J. *Proc. natn. Acad. Sci. U.S.A.* **72**, 3939-3943 (1975).
44. Silverman, M. & Simon, M. *J. Bact.* **130**, 1317-1325 (1977).
45. Matsumura, P., Silverman, M. & Simon, M. *J. Bact.* **132**, 996-1002 (1977).
46. Goy, M. F., Springer, M. S. & Adler, J. *Proc. natn. Acad. Sci. U.S.A.* **74**, 4964-4968 (1977).
47. Springer, W. R. & Koshland, D. E. *Jr Proc. natn. Acad. Sci. U.S.A.* **74**, 533-537 (1977).
48. Van Der Werf, P. & Koshland, D. E. *Jr J. biol. Chem.* **252**, 2793-2795 (1977).
49. Kleene, S. J., Toews, M. L. & Adler, J. *J. biol. Chem.* **252**, 3214-3218 (1977).
50. Stock, J. B. & Koshland, D. E. *Jr Proc. natn. Acad. Sci. U.S.A.* **75**, 3659-3663 (1978).
51. Toews, M. L. & Adler, J. *J. biol. Chem.* **254**, 1761-1764 (1979).
52. Parkinson, J. S. & Revello, P. T. *Cell* **15**, 1221-1230 (1978).
53. Goy, M. F., Springer, M. S. & Adler, J. *Cell* **15**, 1231-1240 (1978).
54. Aswad, D. & Koshland, D. E. *Jr J. Bact.* **118**, 640-645 (1974).
55. Springer, M. S. *et al.*, *Proc. natn. Acad. Sci. U.S.A.* **72**, 4640-4644 (1975).
56. Springer, M. S., Goy, M. F. & Adler, J. *Proc. natn. Acad. Sci. U.S.A.* **74**, 183-187 (1977).
57. Hayashi, H., Koiwai, O. & Kozuka, M. *J. Biochem.* **85**, 1213-1223 (1979).
58. Hazelbauer, G. L. & Harayama, S. *Cell* **16**, 617-625 (1979).
59. Rashevsky, N. *Protoplasma* **20**, 42 (1933).
60. Hill, A. V. *Proc. R. Soc. B119*, 305 (1936).
61. Delbrück, M. & Reichardt, W. in *Cellular Mechanisms in Differentiation and Growth* (ed. Rudnick, D.) 3-44. (Princeton University Press, 1956).
62. Ordal, G. W. *Nature* **270**, 66-67 (1977).
63. Axelrod, J. & Daly, J. *Science* **150**, 892-893 (1965).
64. Liss, M., Maxam, A. M. & Cuprak, L. *J. biol. Chem.* **244**, 1617-1622 (1969).
65. Kim, S. & Paik, W. K. *J. biol. Chem.* **245**, 1806-1813 (1970).
66. Paik, W. K. & Kim, S. *Science* **174**, 114-119 (1971).
67. Morin, A. M. & Liss, M. *Biochem. biophys. Res. Commun.* **52**, 373-378 (1973).
68. Kim, S. *Archs Biochem. Biophys.* **157**, 476-484 (1973).
69. Diliberto, E. J. Jr & Axelrod, J. *Proc. natn. Acad. Sci. U.S.A.* **71**, 1701-1704 (1974).
70. Kim, S., Wasserman, L., Lew, B. & Paik, W. K. *J. Neurochem.* **24**, 625-629 (1975).
71. Diliberto, E. J. Jr & Axelrod, J. *J. Neurochem.* **26**, 1159-1165 (1976).
72. Gallin, J. I. & Quie, P. G. (eds) *Leukocyte Chemotaxis* (Raven, New York, 1978).
73. Schiffmann, E., Corcoran, B. A. & Aswanikumar, S. in *Leukocyte Chemotaxis* (eds Gallin, J. I. & Quie, P. G.) 97-111 (Raven, New York, 1978).
74. O'Dea, R. F. *et al. Nature* **272**, 462-464 (1978).
75. Pike, M. C., Kredich, N. M. & Snyderman, R. *Proc. natn. Acad. Sci. U.S.A.* **75**, 3928-3943 (1978).
76. Diliberto, E. J. Jr, O'Dea, R. F. & Viveros, O. H. in *Transmethylation* (eds Usdin, E., Borchardt, R. T., & Creveling, C. R.) 529-538 (Elsevier, Amsterdam, 1979).
77. Chiang, P. K., Richards, H. H. & Cantoni, G. L. *Molec. Pharmacol.* **13**, 939-947 (1977).
78. Kredich, N. M. & Martin, D. W. *Jr Cell* **12**, 931-938 (1977).
79. O'Dea, R. F. *et al., Fedn Proc.* **37**, 1656 (1978).
80. Gagnon, C., Bardin, C. W., Strittmatter, W. & Axelrod, J. in *Transmethylation* (eds Usdin, E., Borchardt, R. T., & Creveling, C. R.) 521-528 (Elsevier, Amsterdam, 1979).
81. Miller, R. L. *Nature* **254**, 244-245 (1975).
82. Eiden, L. E., Borchardt, R. T. & Rutledge, C. O. in *Transmethylation* (eds Usdin, E., Borchardt, R. T. & Creveling, C. R.) 539-546 (Elsevier, Amsterdam, 1979).
83. Gagnon, C., Viveros, O. H., Diliberto, E. J. Jr & Axelrod, J. *J. biol. Chem.* **253**, 3778-3781 (1978).
84. Koiwai, O. & Hayashi, H. *Abstr. 6th Int. Biophys. Congr., Kyoto* 293 (1978).
85. McGowan, E. B., Silhavy, T. S. & Boos, W. *Biochemistry* **13**, 993-999 (1974).
86. Zukin, R. S., Hartig, P. R. & Koshland, D. E. *Jr Proc. natn. Acad. Sci. U.S.A.* **74**, 1932-1936 (1977).
87. Strange, P. G. & Koshland, D. E. *Jr Proc. natn. Acad. Sci. U.S.A.* **73**, 762-766 (1976).

articles

Riftward younging of volcanic units in the Addis Ababa region, Ethiopian rift valley

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A general riftward younging of surficial volcanic units is a feature of the Addis Ababa sector of the Ethiopian rift valley. The narrowing of the volcanic zone with time is due to crustal necking and topographical control.

THE development of continental rift valleys can be accompanied by a progressive narrowing of the zone of tectonism and volcanism as crustal thinning proceeds¹. In the Ethiopian rift valley, this phenomenon is difficult to discern owing to volu-

minous cover from Quaternary rhyolitic ash-flow and air-fall tuff eruptions. However, the complex and perhaps atypical Addis Ababa embayment² reveals disconnected exposures of older volcanic units with an overall gentle southeastwards dip towards the rift axis³⁻¹². This embayed sector of the western margin of the rift has developed as a broad downwarp lacking any major escarpment; although faulting is common, it is limited to block tilting with associated downthrows to the south-east of tens of metres^{7,8}.

We review here radiometric ages of volcanic rocks along a 100-km-wide swathe, south-east from the Abbay (Blue Nile) basin via Addis Ababa on the rift margin to the rift floor axis at

* Deceased

Table 1 K–Ar age determinations on volcanic samples from the northwestern sector of the Addis Ababa–rift floor traverse

Sample	Locality	Lat (grad N)	Long (grad E)	Analysed portion	% K ₂ O	Vol ⁴⁰ Ar ($\times 10^{-4}$ mm ³ g ⁻¹)	% atmospheric ⁴⁰ Ar	Age (Myr)
Aphanitic basalt (2211)	19 road Km N. of Addis Ababa	10.17	43.06	Whole-rock	1.47	11.17 10.65	56.6 13.3	22.8 $\pm$ 1.5
Porphyritic trachyte (ENT)	North side Entoto, 5 km north of Addis Ababa	10.13	43.07	Felspar	5.31	38.01 37.86	44.6 30.9	22.0 $\pm$ 0.8
Olivine basalt (2213)	North–Central Addis Ababa	10.07	43.07	Whole-rock	0.72	1.69 1.83	65.5 64.6	7.5 $\pm$ 0.6
Basalt (2214)	Northeastern Addis Ababa	10.06	43.09	Whole-rock	0.94	1.95 2.09	64.8 68.2	6.6 $\pm$ 0.5
Basalt (2215)	Southwestern Addis Ababa	9.995	43.010	Whole-rock	3.07	3.71	37.5	3.7 $\pm$ 0.4
Fiammé-rich welded tuff (A1)	Eastern Addis Ababa	10.020	43.080	Feldspar	1.56	2.61 2.56	80.4 92.9	5.11 $\pm$ 0.4
Welded tuff (A2)	Eastern Addis Ababa	10.020	43.085	Felspar	6.28	6.30 6.92	54.7 70.3	3.26 $\pm$ 0.1
Felsphyric basalt (2207)	South side Mt Yerer	9.87	43.30	Whole-rock	2.28	2.60	31.3	3.5 $\pm$ 0.4
Felsphyric basalt (2207)	South side Mt Yerer	9.87	43.30	Feldspar	0.79	1.00	78.7	3.9 $\pm$ 0.4
Trachyte (2208)	South side Mt Yerer	9.87	43.30	Feldspar	3.67	4.34	41.6	3.7 $\pm$ 0.4
Rhyolite (2209)	South side Mt Yerer	9.87	43.30	Feldspar	5.08	5.35	69.2	3.3 $\pm$ 0.4
Porphyritic rhyolite (MEN)	Mt Menegasha tholoid	10.04	42.87	Feldspar	5.75	5.76 5.70	55.6 44.2	3.09 $\pm$ 0.12
Porphyritic trachyte (Z1)	Northeastern base of Mt Zukwala	9.51	43.195	Feldspar	4.14	1.64 1.79	80.4 96.1	1.28 $\pm$ 0.15
Porphyritic trachyte (Z2)	Half-way up northeastern side Mt Zukwala	9.50	43.185	Feldspar	5.52	1.57 1.71	80.9 79.8	0.92 $\pm$ 0.04
Porphyritic trachyte (Z3)	North rim of Zukwala crater	9.493	43.170	Feldspar	5.40	1.41 1.54	88.1 90.6	0.85 $\pm$ 0.05

Samples collected by W.H.M., analysed by D.C.R.

$\lambda_e = 0.581 \times 10^{-10}$ yr⁻¹; $\lambda_\beta = 4.962 \times 10^{-10}$ yr⁻¹; $^{40}\text{K}/\text{K} = 1.167 \times 10^{-2}$ atom %.

Wolenchiti (Fig. 1), and include 33 new K–Ar age determinations on samples critically selected for their position along the rift transect.

Plateau sequence

The plateau basalt sequence exposed along the Abbay gorge road section, 150 km north-west of Addis Ababa, is observed in tributary canyons to thicken southeastwards. The Abbay basalts have yielded K–Ar ages ranging from 27.9 Myr (basal flow) to 24.0 Myr (top flow)¹³. These data are consistent with values obtained by Zanettin and coworkers⁹ on equivalent basalts and a rhyolitic ignimbrite from the upper reaches of the Mugher tributary, but are appreciably older than the 22.1–20.2 Myr age-range obtained by Jones¹⁴ on the same Mugher basalt flows. Maximum ages of 29 Myr have been obtained for basalts from the opposing, eastern shoulder of the rift valley^{11,12}.

On the 2,600 m plateau immediately north of Addis Ababa, the top flood basalt at Sululta yields an apparent age of 22.8 Myr (Table 1), conforming with the Abbay basalt ages and the observed addition of intervening, less far-travelled flows derived from the rift-margin sources¹⁵. Basalts (~13 Myr old) of the Gofu Mts volcanic centre⁹ are built on the plateau.

Embracing Addis Ababa, the arc of the Entotto hills is formed of gently north-dipping comenditic lavas and obsidian-rich tuffs overlying related olivine trachytes^{16,17}. Our date of 22.0 Myr for a northern Entotto lava is concordant with a previous 22.6 Myr age for a southwestern Entotto comendite⁹. These early Miocene silicic volcanics may represent a localised terminal episode to massive Oligocene fissure–basalt activity in the Addis Ababa region, but their stratigraphic relations to the Sululta basalts immediately to the north are not exposed because of a faulted contact. The only other silicic rocks of known similar age occur near the bottom of the Kassam gorge within the rift valley, 40 km east of Entotto, where separate investigations have produced radiometric ages in moderate agreement. Justin-Visentin *et al.*⁹ obtained an age of 23.9 Myr, and Rex *et al.*¹⁸ an age of 21.4 Myr for the lowermost silicic ignimbrite within the

exposed 1,000 m thick Kassam basalt section. (The base of the basalt pile farther down the Kassam gorge has not been sought since Francaviglia's provocative identification there of a restricted window of unfossiliferous, supposedly Mesozoic sediments¹⁹.) The available evidence for early Miocene silicic volcanism in the Addis Ababa region suggests an isolated centre at Entotto, with erupta thinning both riftwards and plateauwards: this centre was later strongly upfaulted as a NE–SW trending horst at the margin of the developing rift.

Above the lower basalts of the Kassam gorge, a 100 m thick trachytic bed at 2,100–2,200 m elevation in the northwestern wall has yielded an age of 16.0 Myr, suggesting a downwarped equivalent to plateau-surface (2,800 m) outcrops in the Debra Berhan region, 60 km farther north-east⁹. Thin rhyolitic ignimbrites of the upper Kassam and Shano area (Fig. 1), with ages in the 13–11 Myr bracket, interdigitate with basalts of the Gofu and Megheze piles⁹, and on the opposing, eastern side of the rift valley, ignimbrites of the same age-range form the 'main silicic formation', up to 300 m in thickness²⁰.

In the environs of Addis Ababa, the oldest visible rocks post-dating the Entotto rhyolites are alkali olivine basalts lapping the southern flanks of the Entotto horst^{4,21}. These very localised basalts yield ages clustering around 7 Myr (Table 1 and ref. 22), and seem to have no time/composition equivalents except on the opposing, eastern shoulder of the rift valley¹¹. However, thin but widespread rhyolitic ignimbrites that occur along the plateau margin north-east of Addis Ababa yield ages in a broad 9.2–7.9 Myr range⁹.

In Addis Ababa town itself, the north-east-trending Filwoha fault separates, to the north, the 7 Myr-old basalts here overlain by pantelleritic welded tuffs from strongly welded trachytic tuffs to the south. The latter yield apparent ages of 5.1 and 3.3 Myr (Table 1). These ages overlap with the periods of activity of two important trachyte volcanoes: Wachacha, 15 km west of Addis Ababa, and Yerer, 25 km south-east of Addis Ababa (Fig. 1). Wachacha erupted a terminal lava phase (thick biotite–anorthoclase trachyte flows) dated at 4.6 Myr^{9,23}. Isolated patches of welded tuff in the Sululta region, dated at 5.4 Myr⁹, as well as the

5.1 Myr welded tuff in Addis Ababa, were probably derived from the Wachacha centre before the Entotto and Filwoha faulting. Likewise, welded tuff exposed in the Akaki river canyon, 25 km south of Addis Ababa and dated at 5.3 Myr, was probably derived from Furi, a satellite of Wachacha.

Yerer volcano lacks the lava cap featuring Wachacha, and its more bare aspect belies the clustering of K-Ar ages at 3.6 Myr (ref. 24 and Table 1). The succession at Yerer is²⁵:

- (4) NW-SE trending basaltic and subordinate trachytic dykes
- (3) Vesicular porphyritic basalts (<20 m), flanking 2
- (2) Thick trachytic agglomeratic tuffs and subordinate lavas
- (1) Massive welded tuffs

In Table 1, the rhyolite and trachyte ages of 3.3 and 3.7 Myr belong to unit (2), and the basalt ages of 3.5 (whole-rock) and 3.9 (plagioclase) Myr to unit (3). A tendency for ⁴⁰Ar migration into plagioclase phenocrysts of Ethiopian rift basalts has been detected by J.G.M. (unpublished data). Contemporaneous activity formed the isolated Managasha tholoid on the northern flanks of Wachacha volcano, but extensive volcanism was now migrating south-east towards the axis of the proto-rift.

For the sector between Bishoftu and Mojo (respectively 45 and 70 km south-east of Addis Ababa) no radiometric ages are available. In contrast to the Addis Ababa-Bishoftu sector, where NE-SW faulting upthrown north-west exposes units derived from Yerer, Wachacha and superimposed end Pliocene-Pleistocene basalt cones^{22,25}, exposures on the plains south-east of Bishoftu are restricted to small silicic centres and young Quaternary basalt cones associated with the Bishoftu maars^{25,26}. At the Mojo River canyon, Pleistocene volcanoclastic and diatomaceous sediments a few metres thick lie on 20 m+ of ignimbrite, but thicken riftwards to 15 m+ (base not seen) in the Melka Lemi canyon 3 km east of Mojo^{27,28}.

At Tedi village, 5 km east of Mojo, the Gadamsa segment of the Wonji fault belt (WFB) is encountered, a zone of closely-spaced, anastomosing normal faulting^{29,30}. The WFB here is 10 km wide and comprises some 12 strip-like blocks, each 700-1,000 m wide, that are tilted down to the ESE at

2-3 grads dips. The separating faults trend NNE and have upthrows to the east averaging about 15 m. However, throws of up to 100 m are responsible for narrow horsts at the margins of the fault belt. The summary stratigraphy within the WFB here is:

- (3) Weakly-welded tuff, thickening and more crystal-rich eastwards (2-4 m)
- (2) Massive pumiceous sediments with occasional well-bedded sub-units and palaeosols (10-20 m)
- (1) Strongly welded tuff, base not exposed (55 m+)

Unit (1), exposed also in the Mojo River canyon, is presumed to have been derived from the Mukie volcanic centre 15 km north of Adama (Fig. 1). Unit (2) thickens both east and west, away from the higher topography of the WFB in the Mojo-Adama sector. On the eastern side of the WFB, feldspar-phyrlic basalts are interbedded near the top of unit (2). They were derived from sources aligned along the subsequently faulted western margin of the Adama graben³⁰, although basaltic eruptions continued within the Adama graben after the deposition of unit (3). Unit (3) resembles unit (2) in being thinner or absent from the higher blocks and horsts of the WFB, but additionally thins out eastwards to zero.

New K-Ar dates for the WFB transect

We present four new K-Ar dates for this WFB transect (Table 2). Unit (3), sampled from the eastern foot of the Kimbabit horst^{22,29}, yielded an apparent age of 0.51 Myr. The basalt flow interbedded in unit (2) was sampled from the 'Stegosaurus' horst overlooking the Adama graben, and yielded an apparent age of 0.61 Myr identical with that of a small olivine basalt flow lapping Gara Boku, 7 km farther south-east. Unit (1) was sampled 4 km east of Tedi and yielded an apparent age of 1.7 Myr, coeval with major silicic volcanism at Boseti Gudda volcano (on the Boseti segment of the WFB, east of Adama) and shield volcanism on the future eastern shoulder of the rift valley^{11,12,31}. However, older rocks floor the rift under the Gadamsa WFB segment, according to the age of porphyritic comenditic obsidian forming the Gara Mariam horst at Tedi (Table 2). Our new date of 3.2 Myr revises previous values for this tholoid²², and proves activity contemporaneous with Yerer and at the eastern, Siri margin of the rift^{11,12}. Gara Mariam, like Entotto, is a discrete silicic igneous centre that has since been upfaulted among younger rocks, in response to isostatic forces acting at the margin of a tensional zone.

Gadamsa volcano with a 10-km-diameter caldera lies on the Gadamsa segment of the WFB some 20 km SSW of Adama³². An age of 0.85 Myr (Table 2) pinpoints the middle period of build-up of the Gadamsa cone. One surprise of our dating program is that an indistinguishable age, 0.83 Myr, applies to a late-stage obsidian lava from the similar-sized Boku caldera situated midway between Gadamsa and Adama (Fig. 1). Boku caldera is so severely broken that it required satellite imagery for its initial recognition^{29,32}. It seems that the caldera-cone diameter ratio at Boku was probably close enough to unity that caldera collapse facilitated the destruction of the greater part of the remnant cone.

Zukwala volcano, 30 km north-west of Gadamsa, lies on an isolated (non-WFB) volcanotectonic line that projects NNE via a Holocene basalt lava cone to the Bishoftu maars³³. The Zukwala trachytes yield a progressive sequence of ages, from 1.28 Myr for a basal exposed flow to 0.85 Myr for a crater rim flow (Table 1).

The Boseti segment of the en-echelon WFB is encountered east of Adama. The geology of this area is dominated by Boseti Gudda volcano^{29,34}, where a caldera is largely buried by a subsequent high cone of pantelleritic tuffs, pumice and subordinate lavas. Morbidelli *et al.*¹¹ obtained a K-Ar age of 1.6 Myr for a near-summit lava, but the fresh morphology of satellite cones and flows on the southern slopes of Boseti Gudda shows that parasitic activity continued into recent times. Boseti Gudda, and its comenditic neighbour Boseti Baricha immediately to the

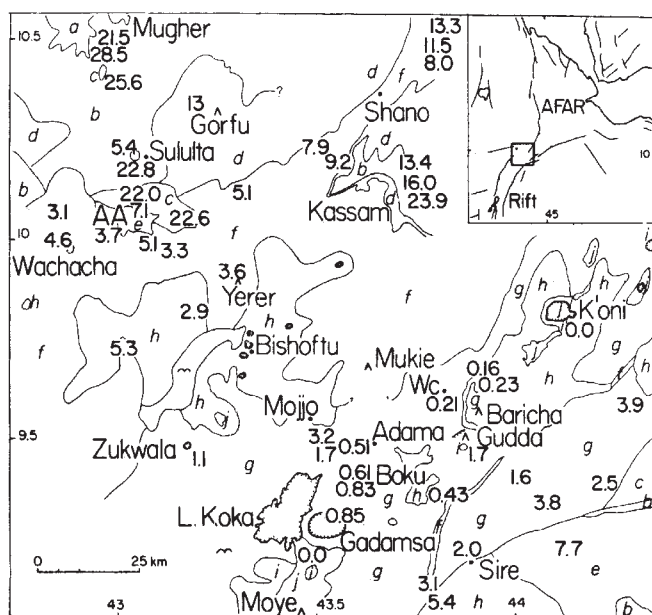


Fig. 1 Chronostratigraphic map of the Addis Ababa region and northern sector of the Ethiopian rift. Numbers are K-Ar ages in Myr. The lettered formations are: a, Mesozoic sediments; b, plateau basalts (older than 22 Myr); c, silicic lavas and tuffs (23-21 Myr); d, shield basalts and subordinate rhyolites (16-13 Myr); e, olivine basalts (8-6 Myr); f, ignimbrites and lavas (11-3 Myr); g, ignimbrites (<3 Myr); h, olivine basalts (<3 Myr); i, Holocene obsidian flows; j, Holocene mafic lavas. AA, Addis Ababa; Wc, Wolenchiti. The detailed fault pattern is omitted. Latitude and longitude in grads provide scale; 1 grad latitude equals 100 km.

Table 2 K-Ar age determinations on volcanic samples from the southeastern sector of the Addis Ababa-rift floor traverse

Sample	Location	Lat (grad N)	Long (grad E)	Analysed portion	% K ₂ O	Vol ⁴⁰ Ar ($\times 10^{-4}$ mm ³ g ⁻¹)	% atmospheric ⁴⁰ Ar	Age (Myr)
Porphyritic obsidian (76-1)	Gara Mariam, Tedi	9.510	43.522	Feldspar	6.15	6.60	39.8	3.32 $\pm$ 0.06
Porphyritic obsidian (76-1)	Gara Mariam, Tedi	9.510	43.522	Matrix (excluding anorthoclase phenocrysts)	5.37	5.41	55.3	3.11 $\pm$ 0.06
Welded tuff (76-3)	3 km south-east of Tedi	9.500	43.541	Whole-rock	4.46	2.45	43.0	1.71 $\pm$ 0.04
Welded tuff (76-5)	Kimbibit horst, 4 km west of Adama	9.483	43.583	Whole-rock	5.10	0.850	91.2	0.51 $\pm$ 0.03
Olivine basalt (76-6)	Scarp, 2 km west of Adama	9.480	43.604	Matrix (excluding labradorite phenocrysts)	0.675	0.135	89.1	0.61 $\pm$ 0.03
Green welded tuff (76-10)	North rim, Gadamsa caldera	9.320	43.556	Whole-rock	0.669 0.810	0.185 0.218	94.8 93.5	0.85 $\pm$ 0.07
Olivine basalt (76-8)	Nagow cone, Gadamsa caldera	9.313	43.557	Whole-rock	1.25	0.168	94.6	0.41 $\pm$ 0.04
Olivine hyalo- basalt (76-15)	Northeastern flanks, Gara Boku	9.417	43.650	Whole-rock	0.920	0.183	89.5	0.61 $\pm$ 0.04
Comenditic obsidian (76-16)	North summit, Gara Boku	9.415	43.645	Whole-rock	4.54	1.21	51.5	0.83 $\pm$ 0.02
Olivine basalt (76-7)	2 km south-west of Bofa	9.395	43.824	Whole-rock	1.28	0.185	90.8	0.44 $\pm$ 0.05
Olivine trachyte (76-12)	Jinjimma flow 1 km south-east of Wolenchiti	9.611	43.808	Whole-rock	4.74	0.325	78.9	0.21 $\pm$ 0.01
Hyalotachyte (76-13)	North flank, Gara Chisa	9.645	43.877	Whole-rock	2.14	0.161	94.4	0.23 $\pm$ 0.03
Aphyric obsidian (76-14)	Summit, Gara Chisa	9.654	43.873	Whole-rock	4.36	0.226	84.4	0.16 $\pm$ 0.01
Olivine mugearite (76-17)	Sogido, 7 km east of Wolenchiti	9.633	43.887	Whole-rock	1.82	0.212	91.1	0.36 $\pm$ 0.03
Holocene hawaiiite flow (RV 337)	Tulu Moye volcanic centre	9.265	43.451	Whole-rock	n.d.	<0.010	100.0	0.0
19th century pyroxene hyalobasalt (G27)	Gariboldi volcanic centre	9.812	44.114	Whole-rock	1.15	<0.016	100.0	0.0
19th century hyalobasalt cinder cone (G203)	Gariboldi volcanic centre	9.81	44.11	Whole-rock	1.05	0.111	95.2	0.31 $\pm$ 0.06

Samples collected by P.M., analysed by J.G.M.
Constants as for Table 1.

north, lie on a line of major crustal fracturing from which mildly alkaline olivine basalts have erupted^{34,35}. An extensive such flow sampled 2 km south of Bofa on the southern flank of Boseti Gudda yields an apparent age of 0.44 Myr (Table 2). Perfectly preserved end-Pleistocene cinder cones lie on a basalt fissure line displaced 7 km west from the Bofa-Boseti line^{35,36}.

On the lower western flanks of Boseti Baricha, the Jinjimma olivine trachyte cone issued a flow which we date at 0.21 Myr, consistent with rather well-preserved morphology^{29,35}. The cone has been broken by a NNE-trending fault with an upthrow of ~200 m to the west²⁹, and smaller parallel faults cut the flow. 6 km farther NNE along this active and intensely sliced zone, trachyte obsidians from the spectacularly faulted and fissured Gara Chisa dome yield ages of 0.23 Myr (northern flank) and 0.16 Myr (summit). The Jinjimma-Chisa line of silicic volcanism thus seems to represent a recent westward side-stepping of silicic volcanism in the WFB. However, young mafic eruptions have continued from the main Boseti alignment, most notably voluminous and highly fluid mugearite flows from the col between Gudda and Baricha^{34,35}. Our date of 0.36 Myr for a mugearite specimen from Sogido (Table 2) is certainly too old, as these lavas are stratigraphically younger than flanking obsidian flows from Gara Chisa dated at 0.23 Myr. If excess argon occurs at a level common to many Ethiopian rift basalts³⁷, then an age of 0.23 Myr would be indicated.

Conclusions

In summary, a general riftward younging of surficial volcanic units is a clear feature of the Addis Ababa sector of the Ethiopian rift valley. Exceptions to this pattern are interesting but second-order phenomena, such as the Zukwala volcanic line along the western margin of the rift, and young centres on the

plateau west of Addis Ababa^{27,40}. The younging phenomenon is consonant with the broad crustal downwarp developed between the Abbay basin and the rift axis^{38,39}. Downwarping towards the future site of the rift valley began in late Jurassic time, continued throughout Oligocene-Miocene flood basalt extrusion, and was active together with block faulting during early-mid Pleistocene differential uplift of the Ethiopian plateaux.

But does the progressive confining of rift volcanism with time merely reflect topographic control, from deepening of the proto-rift trough that culminated in mid-Pleistocene downfaulting of the rift valley? Or does it reflect a fundamental control of eruptive sites from processes of crustal necking and separation⁴¹? Plate tectonic analysis of the Afar triple-junction has led to two estimates of Ethiopian rift 'opening' of 65 km (ref. 42) and 40 km (ref. 43). The latter better fits the geological and geophysical constraints. Geologically, there is continuous exposure of ~24 Myr-old volcanic units for at least 20 km riftwards down the Kassam gorge; and at the southern end of the Ethiopian rift, the floor is severely faulted but exposes Precambrian rocks⁴⁴. Geophysically, crustal thickening occurs east across the Ethiopian plateau to Addis Ababa and possibly as far as Bishoftu^{45,46}; however, gravity modelling of the crust within the rift implies drastic thinning and an extension of 25-40 km if stopping processes are ruled out^{47,48}.

Elucidation of the cause of progressive confinement is complicated by the changing character of rift volcanism with time, from voluminous flood basalt lavas through episodic but intimate mafic and silicic eruptions, to relatively voluminous silicic tuffs. Furthermore, earlier eruptive sites are now hidden. We conclude, on the basis of our work together with known geophysical constraints, that narrowing of the volcanic zone in the Ethiopian rift with time is due partly to crustal necking, but more significantly to topographical control⁴⁹.

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1. Baker, B. H., Crossley, R. & Goles, G. G. in *Petrology and Geochemistry of Continental Riffs*, 29–50 (Reidel, Dordrecht, 1978).
2. Mohr, P. A. *Bull. geophys. Obs. Addis Ababa* **11**, 1–67 (1967).
3. Norconsultants, *Report* (Ethiopian Electric Light and Power Authority, Addis Ababa, 1957).
4. Kondo, C. *Rep. Res. Inst. Underground Resources, Akita Univ.* **19**, 20–40 (1958).
5. Dept of Geology, *Student mapping projects* (Addis Ababa University, 1972–1976).
6. Mohr, P. A. *Bull. geophys. Obs. Addis Ababa* **4**, 65–101 (1961).
7. Mohr, P. A. *Bull. geophys. Obs. Addis Ababa* **10**, 99–112 (1967).
8. Habte Giorgis Churnet, H.S.I. Univ. Dept of Geology, A. Rep. 1973–74, 20–21 (1974).
9. Justin-Visentin, E., Nicoletti, M., Tolomeo, L. & Zanettin, B. *Bull. Volc.* **38**, 237–253 (1974).
10. Zanettin, B. *et al. Neues Jb. Geol. Paläont. Ref. 9*, 567–574 (1974).
11. Morbidelli, L., Nicoletti, M., Petrucciani, C. & Piccirillo, E. M. *Inter-Un. Commn. Geodynam. Sci. Rep.* **14**, 362–369 (1975).
12. Kunz, K., Kreuzer, H. & Müller, P. *Inter-Un. Commn. Geodynam. Sci. Rep.* **14**, 370–374 (1975).
13. McDougall, I., Morton, W. H. & Williams, M. A. J. *Nature* **254**, 207–209 (1975).
14. Jones, P. W. *Nature* **261**, 567–569 (1976).
15. Mohr, P. A. *Bull. geophys. Obs. Addis Ababa* **6**, 103–144 (1963).
16. Hieke-Merlin, O. *Mem. Inst. Geol. Min. Univ. Padova*, **18** (1953).
17. Gortani, M. & Bianchi, A. *Accad. Naz. Lincei. Roma*, Vol. 1 (1973).
18. Rex, D. C., Gibson, I. L. & Dakin, F. *Nature phys. Sci.* **230**, 131–132 (1971).
19. Francaviglia, A. *Boll. geol. Soc. Ital.* **59**, 205–220 (1940).
20. Juch, D. *Clausthaler Geol. Abh.* **29** (1978).
21. Comucci, P. *Proc. verb. Soc. Toscana Sci. Nat. Pisa* **41**, 100–111 (1932).
22. Mohr, P. A. *Smithson. Astrophys. Obs. Spec. Rep.* **358** (1974).
23. Miller, J. A. & Mohr, P. A. *Bull. geophys. Obs. Addis Ababa* **9**, 1–5 (1966).
24. Morton, W. H. & Rex, D. C. *Bull. geophys. Obs. Addis Ababa* **15**, 156 (1975).
25. Mohr, P. A. *Bull. geophys. Obs. Addis Ababa* **10**, 99–112 (1967).
26. Mohr, P. A. *Bull. geophys. Obs. Addis Ababa* **4**, 65–101 (1961).
27. Gouin, P. & Mohr, P. A. *Bull. geophys. Obs. Addis Ababa* **7**, 185–236 (1964).
28. Taieb, M. thesis, Univ. Paris VI (1974).
29. Mohr, P. A. *Smithson. Astrophys. Obs. Spec. Rep.* **376** (1977).
30. Mohr, P., Girnius, A. & Rolff, J. *Tectonophysics* **44**, 141–160 (1978).
31. Di Paola, G. M. *Geological Map of the Tullu Moye Volcanic Area* (Cons. Naz. Ricerche, Pisa, 1976).
32. Thrall, R. *Bull. geophys. Obs. Addis Ababa* **15**, 71–81 (1975).
33. Mohr, P. A. & Wood, C. A. *Earth planet. Sci. Lett.* **33**, 126–144 (1976).
34. Brotzu, P., Morbidelli, L., Piccirillo, E. M. & Traversa, G. *Bull. Volc.* **38**, 206–234 (1974).
35. Brotzu, P., Morbidelli, L., Piccirillo, E. M. & Traversa, G. *Geological Map of Boseti Volcanic Complex* (Cons. Naz. Ricerche, Roma, 1978).
36. Meyer, W., Pilger, A., Rösler, A. & Stets, J. *Inter-Un. Commn. Geodynam. Sci. Rep.* **14**, 352–362 (1975).
37. Mohr, P., Mitchell, J. G. & Raynolds, R. G. H. *Bull. Volc.* (in the press).
38. Jepsen, D. H. & Athearn, M. J. *East-West Geologic Sections, Blue Nile river basin, Ethiopia* (Dept. water resources, Addis Ababa, drawing 5.2 BN-3, 1962).
39. Mohr, P. A. *Bull. geophys. Obs. Addis Ababa* **5**, 33–62 (1962).
40. Le Bas, M. J. & Mohr, P. A. *Geol. Rdsch.* **58**, 273–280 (1968).
41. Baker, B. H. & Mitchell, J. G. *J. geol. Soc. Lond.* **132**, 467–484 (1976).
42. McKenzie, D. P., Davies, D. & Molnar, P. *Nature* **226**, 1–6 (1970).
43. Le Pichon, X. & Francheteau, J. *Tectonophysics* **46**, 369–406 (1978).
44. Levitte, D., Columba, J. & Mohr, P. *Bull. geol. Soc. Am.* **85**, 417–422 (1974).
45. Searle, R. C. & Gouin, P. *Bull. seis. Soc. Am.* **61**, 1061–1071 (1971).
46. Berckheimer, H. *et al. in Afar Depression of Ethiopia*, 89–107 (Schweizerbart, Stuttgart, 1975).
47. Searle, R. C. & Gouin, P. *Tectonophysics* **15**, 41–52 (1972).
48. Baker, B. H., Crossley, R. & Goles, G. G. in *Petrology and Geochemistry of Continental Riffs*, 29–50 (Reidel, Dordrecht, 1978).
49. Williams, L. A. J. *Inter-Un. Commn. Geodynam. Sci. Rep.* **39**, 101–121 (1978).

Sequences at the somatic recombination sites of immunoglobulin light-chain genes

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The entire nucleotide sequence of a 1.7-kilobase embryonic DNA fragment containing five joining (J) DNA segments for mouse immunoglobulin κ chain gene has been determined. Each J DNA segment can encode amino acid residues 96–108. Comparison of one of the five J DNA sequences with those of an embryonic variable (V) gene and a complete κ chain gene permitted localisation of a precise recombination site. The 5'-flanking regions of J DNA segments could form an inverted stem structure with the 3'-non-coding region of embryonic V genes. This hypothetical structure and gel-blotting analysis of total embryo and myeloma DNA suggest that the somatic recombination may be accompanied by excision of an entire DNA segment between a V gene and a J DNA segment. Antibody diversity may in part be generated by modulation of the precise recombination sites.

IMMUNOGLOBULIN polypeptide chains consist of two regions, the amino-terminal half, called the variable (V) region, and the carboxyl-terminal half, called the constant (C) region. In embryonic cells the two regions are encoded in widely separated DNA segments. During differentiation of bone marrow-derived (B) lymphocytes the two DNA segments are brought into proximity by site-specific recombination, producing a single transcription unit^{1,2}.

The details of the sequence organisation before and after recombination have been worked out in the mouse λ I light-chain system by analysis of cloned DNA fragments^{3,4}. These studies revealed that in embryo cells the conventionally defined

V region is itself encoded in two separate DNA segments, one (V DNA) coding for the major part of the V region (residues 1–97) and the other (J DNA) coding for the rest of the V region (residues 98–109). Although the distance between the V and the C DNA segments is unknown, the J DNA segment has been mapped at 1.2 kilobases towards the 5'-side, relative to the direction of transcription, of the C DNA segment.

Somatic recombination takes place at the 3'-end of the V DNA segment and the 5'-end of the J DNA segment. Consequently, in a λ I chain synthesising B lymphocytes or plasma cells, although the DNA segment coding for the entire V region is much closer to the C DNA segment, the two DNA segments are still separated by 1.2 kilobases. This untranslated sequence (intron)⁵ separating the J and C DNA segments is thought to be removed at the RNA level by a process called splicing^{6,7}. Hence, the J DNA segment and its flanking sequences seem to be involved in two key processes essential for the expression of an immunoglobulin gene: site-specific V–J recombination and J–C splicing³.

More recently, we have analysed the organisation of mouse κ light-chain genes^{8,9}. A comparison of the sequence organisation of the two light-chain gene systems revealed both common and differing features. As in the λ gene system, a secreted κ chain is encoded in three separate DNA segments in embryonic cells, V κ , J κ and C κ . In addition, somatic recombination takes place between embryonic V κ and J κ DNA segments. However, unlike the λ I gene system, the mouse genome contains multiple V κ and J κ DNA segments, the latter being clustered in the vicinity of a single copy of the C κ DNA segment.

For a better understanding of the structural features of the recombination sites we have determined the nucleotide sequences of the J κ cluster as well as the 3'-end region of an embryonic V κ DNA. This information, in addition to Southern

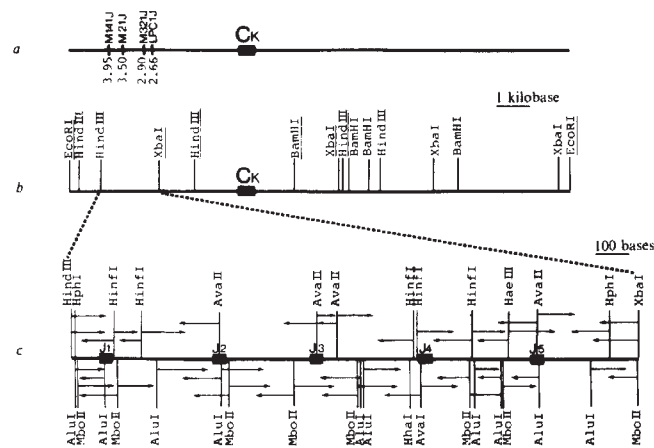


Fig. 1 *a*, R-loop map of the 15-kilobase insert of Ig146. κ mRNAs from four different myelomas, MOPC141, MOPC21, MOPC321 and LPC1, were individually annealed with the Ig146 *EcoRI* DNA fragment and the position of the corresponding J DNA segment was determined by electron microscopy (see ref. 9 for details). The bars represent the J DNA segments. The numbers indicate their distance from the $C\kappa$ DNA segment. *b*, Restriction enzyme cleavage map of the 15-kilobase insert of Ig146. The whole Ig146 phage DNA and the purified vector phage (λ WES)³⁰ arm DNA (1 μ g each) were digested either with one of the indicated enzymes or with that enzyme plus *EcoRI*, and separated by electrophoresis in a 1% agarose gel. Comparison of the single and double digestion patterns allowed localisation of the outermost cleavage sites of each enzyme on the 15-kilobase insert. Other cleavage sites were determined by comparing the pattern obtained by a single enzyme digestion (enzyme A or B) with that obtained by a double enzyme digestion (enzyme A plus B). The position of the $C\kappa$ DNA segment relative to the underlined cleavage sites was determined by examination of R-loop structures formed between MOPC321 κ mRNA and the Ig146 DNA predigested with the corresponding enzyme. *c*, Sequencing strategy and a restriction map of the 1.7-kilobase *HindIII*-*XbaI* fragment. The rightmost cleavage site of each enzyme was identified by comparing the cleavage pattern of the 1.7-kilobase *HindIII*-*XbaI* fragment with that of the 2.7-kilobase *HindIII*-*HindIII* fragment. For determination of additional cleavage sites the *HindIII*-*XbaI* fragment was end-labelled with [γ -³²P]ATP and separated into two fragments by *HhaI* digestion followed by electrophoresis. The purified *HindIII*-*HhaI* and *HhaI*-*XbaI* fragments were digested with one of the indicated enzymes in limited conditions and the cleavage sites were deduced according to the method of Smith and Birnstiel³¹. Horizontal arrows indicate the direction and the extent of sequence determination. The boxes indicate positions of J DNA segments as determined by the nucleotide sequence analysis.

gel-blotting experiments, was used to formulate a model for somatic recombination. We also discuss V-J joining with respect to generation of antibody diversity and evolution of immunoglobulin genes.

Sequencing strategy for the J DNA cluster

We have previously reported cloning of a 15-kilobase embryonic DNA fragment (clone Ig146 κ) containing a single $C\kappa$ DNA copy⁸. Analyses of R-loops formed between this DNA fragment and five different κ mRNAs isolated from myelomas MOPC141, MOPC21, MOPC321, LPC1 and HOPC8 identified at least four different J DNA segments (HOPC8 J and LPC1 J may be identical) in the region 2.5–4.0 kilobases to the left of the $C\kappa$ DNA segment (Fig. 1*a*)⁹. To determine the nucleotide sequence of this region, we first constructed a restriction map of the 15-kilobase Ig146 κ DNA using restriction enzymes *HindIII*, *BamHI*, and *XbaI* (Fig. 1*b*). The position of the $C\kappa$ DNA segment in the restriction map was determined by examining R-loops formed between MOPC321 κ mRNA and the Ig146 κ DNA predigested with the enzymes listed above. From a comparison of this restriction map with the R-loop map previously constructed for J segments we concluded that the J DNA cluster is on the 1.7-kilobase *HindIII*-*XbaI* fragment (Fig. 1*a,b*). We then determined the entire nucleotide sequence of this fragment using a strategy shown in Fig. 1*c*.

Complete nucleotide sequence of the J-rich 1.7-kilobase *HindIII*-*XbaI* fragment

Figure 2 shows the complete nucleotide sequence of the *HindIII*-*XbaI* fragment. To date, about 30 mouse κ chains of BALB/c and NZB strains have been sequenced around the J region^{10,11}. If one assumes that the $J\kappa$ region begins with residue 96 and ends with residue 108 (see below) based on the analogy to the $J\lambda$ region⁴, the 30 $J\kappa$ regions fall into 10 different classes (Table 1). We therefore scanned the nucleotide sequence determined for each of the 10 predicted J sequences, and identified four (J1, J2, J4 and J5). Taking advantage of the fact that all known J regions are extensively homologous, we looked for additional J or J-like sequences and found one (J3) for which no sequenced κ chain is known. A systematic computer search for other J-like sequences that are related to the known or predicted J sequences by more than 50% homology revealed no more than the five Js in the 1.7-kilobase *HindIII*-*XbaI* fragment.

The amino acid sequences of the MOPC21 and MOPC321 κ chain J regions correspond to the predicted sequences of J2 and J4, respectively. Positions of these Js as determined by sequencing agree well with the positions of Js identified by R-loop mapping using the respective κ mRNA. We therefore conclude that J2 and J4 encode MOPC21 and MOPC321 J regions, respectively. Amino acid sequences of the MOPC141 and LPC1 (or HOPC8) J regions are unknown, but based on the R-loop map (Fig. 1*a*) we predict that these J regions are encoded in J1 and J5, respectively.

The distance between adjacent Js is quite constant. The five Js are arranged as follows: J1-310 base pairs-J2-246 base pairs-J3-268 base pairs-J4-299 base pairs-J5. We have recently completed sequencing a stretch of about 1 kilobase on each side of the *HindIII*-*XbaI* fragment and have found no J or J-like sequences. If all Js are clustered and regularly arranged every 300 or so base pairs, there should be no J sequences other than those identified here. On the other hand, if all J peptides (see Table 1) are germ-line encoded, there should be additional J DNA segments elsewhere. Alternatively, some J peptides may be somatically generated (see below).

Precise boundaries of J DNA segments

Our previous nucleotide sequencing studies of embryonic V λ , embryonic C λ and myeloma V λ + C λ DNA clones identified the site of V-J recombination within one of two alternative pairs of phosphodiester bonds⁴. To obtain similar information for a κ chain gene, it is necessary to identify a germ-line V DNA of a specific κ chain for which the J DNA sequence is known, and determine the nucleotide sequences of both germ-line V DNA and myeloma (V + C) DNA. To this end the germ-line V gene of MOPC321 κ chain, V κ 21-C, was cloned and sequenced (details to be published elsewhere). We also determined the partial nucleotide sequence of the cDNA clone of MOPC321 κ mRNA, 5D10 (ref. 3). Nucleotide sequences around the putative recombination sites on V κ 21-C and J4 (which encodes the $J\kappa$ peptide of MOPC321 κ chain, see above) as well as the sequence of the corresponding region of 5D10 are:

	95
5D10	AsnGlnAspProPheThrPheGly AATGAGGATCCATTCACGTTCCGGC
V κ 21-C	AATGAGGATCCCTCCACAGTGGCT
J4	GAATCACTGTGATTACAGTTCCGGC

Examination of these sequences enabled us to conclude that the recombination is at the phosphodiester bond linking the second and third nucleotides of the proline codon at residue 95. This corresponds to residue 97 in the λ chain numbering system. In the case of one λ gene we have shown that recombination occurs either between the His(97) and Phe(98) codons or between the

1 HindIII AAGCTTTCGACGTACCCACTGCTCTCTCTTCAGTGAGGAGGGTTTTTGTACAGCCAGACAGTGGAGTACTACCAC
TTCGAAAGCGTCGATGGGTGACGAGACAAGGAGAAGTCACTCCTCCCAAAACATGTCGGTCTGTCACTCATGATGGT 80

J1 *TrpThrPheGlyGlyGlyThrLysLeuGluIleLysArg*
TGTGGTGGACGTTCCGTTGGAGGACCAAGCTGGAAATCAAACGTAAGTAGAATCCAAAGTCTCTTCTCCGTTGTCTATGTCTGTGGCTTCTATGTCTAAAAATGATGTATAAAATCTT
ACACCACTGCAAGCCACCTCCGTGGTTGACCTTTAGTTTGCATTATCTTAGGTTTCAGAGAAAGAGGCAACAGATACAGACCCGAAGATACAGATTTTACTACATATTTTAGAA 200

ACTCTGAAACCAAGATTCTGGCACTCTCCAAGGCAAGATACAGAGTAACCTCCGTTAAGCAAAGCTGGGAATAGGCTAGACATGTTCTCTGGAGAATGAATGCCAGTGTAAATAATTAACA
TGAGACTTTGGTTCTAAGACCGTGAGAGGTTCCGTTTCTATGTCTATTGAGGCAATTTCGTTTCGACCCTTATCCGATCTGTACAAGAGACCTCTTACTTACGGTCACTATTATTAATTGT 320

CAAGTGATAGTTTCAGAAATGCTCAAAGAAGCAGGGTAGCCTGCCCTAGACAAACCTTTACTCGGTGCTCAGACCATGCTCAGTTTTTGTATGGGGGTTGAGTGAAGGGACACCACTGTG
GTTCACTATCAAAGTCTTTACAGATTTCTTCGTCCTATCGGACGGGATCTGTTTGGAAATGAGCCACGAGTCTGGTACGAGTCAAAAACATACCCCACTCACTTCCCTGTGGTCACAC 440

J2 *TyrThrPheGlyGlyGlyThrLysLeuGluIleLysArg*
TGTACACGTTCCGAGGGGGGACCAAGCTGGAAATCAAACGTAAGTAGTCTTCTCAACTCTGTTTCACTAAGTCTAACCTTGTAAAGTTGTTCTTGTGTGTGTTTTTCTTAAGGAGATT
ACATGTGCAAGCCCTCCCTCCGTGGTTGACCTTTATTTTGCATTATCTAGAAGAGTTGAGAACAGTATTGAGATTGGAACATTCACACAGAAACACACACAAAAAGAAATCTCTCTAA 560

TCAGGGATTTAGCAATCCATCTCAGATCAAGTGTAAAGGAGGAAAACTGCCCAAGAGGTTGGAATGATTTTTCAGGCTAAATTTTAGGCTTTCTAAACCAAGTAACCTAACTAGGG
AGTCCCTAAATCGTTTAGGTAGAGTCTAGTTCAACAATCTCCCTTTTACGCGGTGTTCTCCAACCTTACTAAAGTCGATTTAAATCCGAAAGATTGGTTTTCATTGATTGATCCC 680

J3 *IleThrPheSerAspGlyThrArgLeuGluIleLysPro*
GAAGAGGGATAATGTCTACCTAGGAGGGTTTTTGTGGAGGTAAAGTTAAATAAATCACTGTAAATCACATTCAGTGTGGCACCAGACTGGAAATAAACCTTAAGTACATTTTGTCTC
CTTCTCCCTATTAACAGATGGATCCCTCCCAAAACCTCCATTTCAATTTTATTTAGTGACATTTAGTGTAAAGTCACTACCTGGTCTGACCTTTATTTTGGATTATGTAAAAACGAG 800

AACTGCTGTGAAGTTTGGTCCATTGTGCTTTGTATGAGTTTGTGGTGTACATTAGATAAATGAATATTCCTTGTAAACCAAACTTAAATAGAAGAGAACCAAAATCTAGCTA
TTGACGAACACTTCAAACACAGGTAACACAGAAACATACTCAACACCACATGTAATCTATTTACTTGTAAAGAACATTGGGTTTTGAATTTATCTCTCTTGGTTTTTAGATCGAT 920

CTGTACAAGCTGAGCAACAGACTGACCTCATGTCTCAGATTGTGGGAGAAATGAGAAAGAACAGTTTTTCTCTGAACCTTAGCCATCTAACTGGATCAGCCTCAGGCAGGTTTTGTAA
GACATGTTGCACTCGTTTGTCTGACTGGAGTACAGTCTAAACACCTCTTTACTCTTTCTTGTCAAAAAGAGACTTGAATCGGATAGATTGACCTAGTCGGAGTCCGTCACAAACATT 1040

J4 *PheThrPheGlySerGlyThrLysLeuGluIleLysArg*
AGGGGGGCGCAGTGATATGAATCACTGTGATTCACGTTCCGGCTCGGGGACAAAGTTGGAAATAAACGTAAGTAGACTTTTGTCTATTACTTGTGACGTTTTGGTTCTGTTTGGGTAAC
TCCCCCGCGTCACTATACTTAGTGACACTAAGTGCAAGCCGACCCCTGTTTCAACCTTTATTTTGCATTATCTGAAACGAGTAAATGAACACTGCAAAACCAAGCAAAACCCATTG 1160

TTGTGTGAATTTGTGACATTTTGGCTAAATGAGCATTCTAGCAACCTGTGCATCAATAGAAGATCCCCAGAAAAGAGTCACTGTGAAAGCTGAGCGAAAACTCGTCTTAGGCTTCT
AACACACTTAAACACTGTAAACCGATTACTCGGTAAAGATCGTTGGACAGTATGTTATCTTAGGGGTCTTTTCTCAGTCACACTTTTCGACTCGCTTTTGTAGCAGAAATCCGAAGA 1280

GAGACAGTTTGTAAAGGGGAATGTAGAAGAAAGAGCTGGGCTTTTCTCTGAAATTTGGCCATCTAGTTGGACTGGCTTCACAGGCAGGTTTTTGTAGAGAGGGGATGTCATAGTCTCT
CTCTGGTCAAAACATTCCTCTTACATCTTCTTCTGACCCGAAAAGGAGACTTAAACCGGGTAGATCAACCTGACCGAAGTGTCCGTCCAAAAACATCTCTCCCGTACAGTATCAGGA 1400

J5 *LeuThrPheGlyAlaGlyThrLysLeuGluLeuLysArg*
CACTGTGGCTCAGCTTCGGTGTGGGACCAAGCTGGAGCTGAACGTAAGTACACTTTTCTCATCTTTTTTATCTGTGAAGACACAGGTTTTCATGTTGGAGTTAAAGTCAAGTTCAGAAA
GTGACACCGAGTGCAAGCCACGACCTGGTTGACCTCGACTTGCATTATCTGTAAAGAGTAGAAAAAATAGACATCTCTGTGTCACAAAGTACACCTCAATTTTCAGTCAAGTCTTT 1520

ATCTTGAGAAATGGAGAGGGCTCATTATCAGTTGACGTGGCATACTAGTGTGAGATTTTCTGTTTATATCAGCTAGTGAGATTAGGGGCAAAAGAGGCTTTAGTTGAGAGGAAAGTAAT
TAGAAGCTCTTTTACCTTCCGAGTAATAGTCAACTGCACCTATGTACAGTCTAAAGAGACAAATATAGTCTGATCACTTAATCCCGTTTCTTCCGAAATCAACTCTCCTTTTCATTA 1640

TAATCTATGTTGACCATCAAGAGATTGGATCGGAGAATAAGCATGAGTAGTTATTGAGATCTGGGTCTGACTGCAGGTAGCGTGGTCTCTCTAGA XbaI 1736
ATTATGATACAGTGGTAGGTTCTCTAACCTAGCCTCTATTCTGACTCATCAATAACTCTAGACCCAGACTGACGTCCATCGCACCAGAAGATCT

Fig. 2 Complete nucleotide sequence of the 1.7-kilobase *HindIII*-*XbaI* fragment containing the J cluster. For sequencing, a 2.7-kilobase *HindIII*-*HindIII* fragment carrying the J cluster (Fig. 1b) was subcloned into a plasmid vector pBR322 (ref. 32). A 300- μ g portion of the chimaeric plasmid DNA was digested with a mixture of *HindIII* and *XbaI* and separated in a 6% polyacrylamide gel (20 $\times$ 40 $\times$ 0.5 cm); the 1.7-kilobase *HindIII*-*XbaI* fragment (Fig. 1b) was then purified. A 5- μ g aliquot of this fragment was digested with *HinfI*, *MboII*, *AluI*, *AvaI*, *HphI*, *HaeIII* or *AvaII*, end-labelled and sequenced by the method of Maxam and Gilbert³³ as described previously²⁶. In some experiments, 3'-ends of the DNA fragments were labelled with [α -³²P]nucleoside triphosphates (NTPs) using the Klenow fragment of *Escherichia coli* DNA polymerase I (ref. 34). For this, 1 μ g of DNA was incubated with 20 μ Ci each of four [α -³²P]-NTPs (3,000 Ci mmol⁻¹, NEN) and 1 unit of the Klenow enzyme (Boehringer, Mannheim) at 25 $^{\circ}$ C for 20 min in 50 μ l of 6 mM Tris-HCl, pH 7.5, 50 mM NaCl, 6 mM MgCl₂ and 6 mM β -mercaptoethanol. Aliquots (50 mM) of each of the four NTPs and 0.5 unit of the enzyme were then added to the reaction mix and the incubation was continued for another 10 min. The reaction was terminated by heating the mixture at 70 $^{\circ}$ C for 10 min and DNA was precipitated by ethanol. For strand separation the labelled DNA samples were dissolved in 20 μ l of 50% (v/v) dimethyl sulphoxide, 10 mM Tris-borate, pH 8.3, 1 mM EDTA, 0.5% (v/v) xylene cyanol and 0.05% (v/v) bromophenol blue, heated at 90 $^{\circ}$ C for 3 min, chilled on ice and fractionated in a 6% or 8% strand-separation gel (bisacrylamide/acrylamide = 1/62.5) (A. Maxam, personal communication). Amino acid sequences predicted from the identified J DNA segments are shown in italics.

first and second bases of the latter codon⁴. Thus, the exact sites of recombination in the two cases seem to differ by one or two bases. It remains to be seen whether this difference is due to characteristics of the two recombination systems, one for κ chains and the other for λ chains. An alternative possibility is that the recombinases allow a limited flexibility in the sites of breakage and reunion regardless of the chain type (see below). In this respect it is relevant that all mouse κ chains sequenced to date carry proline at residue 95. As none of the Js reported here can code for this proline (Fig. 2) it is very likely that the first residue fully encoded in J is always at position 96. However, precise recombination sites can only be defined by comparative sequencing analysis of the three DNA segments involved.

Where does coding by J end? The sequences of J4 and 5D10 in the relevant regions are as follows:

	108
5D10	<i>LeuGluIleLysArgAlaAspAla</i> TTGGAAATAAAACGGGCTGATGCT
J4	TTGGAAATAAAAC GTAAGTAGACT

These sequences indicate that J4 can code for MOPC321 κ chain up to the second base of the Arg(108) codon, CGG. However, as J is followed by an intron preceding the C DNA segment and as most introns start with GT (ref. 12), we suggest that coding by J actually ends with the first base of the triplet CGT, as indicated by the vertical line. As shown in Fig. 3, sequences around the splice site are highly conserved among the five Js. In all cases except for J3, for which no κ chains are

Table 1 Classification of BALB/c and NZB myeloma κ chains

Group	Myeloma					Jκ Peptide													Jκ DNA Segment
I	M141*	M63	B32	2880	7210	96 Trp	Thr	Phe	Gly	Gly	Gly	Thr	Lys	Leu	Glu	Ile	Lys	108	J1
	M70	M41	A17	1229	6308													Arg	
	C101	T111	X-44	7769															
II	M21	A22	9245	3741		<u>Tyr</u>	Thr	Phe	Gly	Gly	Gly	Thr	Lys	Leu	Glu	Ile	Lys	Arg	J2
III	M173	6684				<u>Arg</u>	Thr	Phe	Gly	Gly	Gly	Thr	Lys	Leu	Glu	Ile	Lys	Arg	
IV	M11	7940				<u>Pro</u>	Thr	Phe	Gly	Gly	Gly	Thr	Lys	Leu	Glu	Ile	Lys	Arg	
V	T124					<u>Trp</u>	Thr	Phe	Gly	<u>Ser</u>	Gly	Thr	Lys	Leu	Glu	Ile	Lys	Arg	
VI	M321	7043				<u>Phe</u>	Thr	Phe	Gly	<u>Ser</u>	Gly	Thr	Lys	Leu	Glu	Ile	Lys	Arg	J4
VII	X-24					<u>Ile</u>	Thr	Phe	Gly	<u>Ser</u>	Gly	Thr	Lys	Leu	Glu	Ile	Lys	Arg	
VIII	LPC1*	M511	4045	3154		<u>Leu</u>	Thr	Phe	Gly	<u>Ala</u>	Gly	Thr	Lys	Leu	Glu	<u>Leu</u>	Lys	Arg	J5
	HOPC8*	7175	2485	7183															
IX	T601					<u>Ile</u>	Thr	Phe	Gly	<u>Ala</u>	Gly	Thr	Lys	Leu	Glu	<u>Leu</u>	Lys	Arg	
X	2413					<u>Trp</u>	Thr	Phe	Gly	<u>Gly</u>	Gly	Thr	<u>Asp</u>	Leu	Glu	<u>Ile</u>	<u>Glu</u>	Arg	
XI						<u>Ile</u>	Thr	Phe	<u>Ser</u>	<u>Asp</u>	Gly	Thr	<u>Arg</u>	Leu	Glu	Ile	Lys	<u>Pro</u>	J3

BALB/c and NZB myeloma κ chains are classified into 10 groups according to the amino acid sequence of the J κ peptide (residues 96–108)^{10,11}. The DNA segments coding for four J κ peptides, J1, J2, J4 and J5, have been identified on the embryonic C κ clone, Ig146 κ , in the present study. No myeloma κ chain is known for the fifth J DNA segment, J3, also identified in the present study. Those amino acid residues different from the corresponding residues of the J1 peptide are underlined.

* Myelomas classified by R-loop mapping rather than by amino acid sequencing (see text).

known, the doublet GT is present in the last J triplet (CGT) that can partially encode κ chains. It is likely, therefore, that coding by J always ends with the first base of this triplet. In the equivalent position of J3 a Pro codon, CCT, replaces the Arg codon, CGT. It is not clear whether J3 is a genuine J, and if so, where the coding precisely ends.

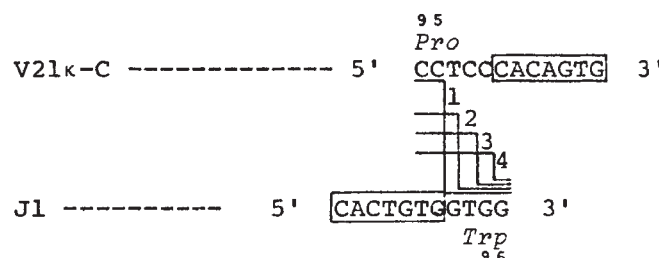
Inverted repeat-stem structure could be formed between embryonic V and J sequences

Figure 3 compares the sequences of five Js in the coding and flanking regions. Sequences are highly conserved in the coding regions and around the RNA splice sites. In contrast, sequences in the rest of the flanking regions are rather diverse, although the non-coding sequences between J3 and J4, and J4 and J5 are somewhat similar. Nevertheless, there are two short conserved sequences in the 5'-non-coding regions. One is a decamer, GGTTTTGTGA, located about 30 base pairs away from all Js, and the other is a palindromic hexamer interrupted by an AT base pair at the centre of symmetry, CACTGTG, immediately preceding the Js. The same or closely related sequences are also present at the equivalent position in the λ I chain gene clone⁴.

The significance of these conserved sequences with respect to V–J joining became apparent when the sequences of an embryonic V κ gene clone, IgV κ 21-C, and an embryonic V κ I clone, Ig99 λ (ref. 4), were examined. Both V DNA clones contain sequences closely related to the decamer and heptamer described above on the anti-sense strands in the 3'-non-coding regions. The presence of these sequences could permit the formation of an inverted repeat stem structure between the 3'-end of an embryonic V sequence and the 5'-end of a J sequence. Examples of such stem structures are shown in Fig. 4a–d. V κ 21-C can form a stem structure of similar stability with any one of the five Js. Another V κ gene, VK-2 (ref. 13), which codes for a V region remotely related to the MOPC321 V region, can also form such a stem structure with any one of the five Js (Fig. 4c). We note that the majority of bases conserved in the 3'-non-coding regions of the two V κ genes participate in base pairing with Js. A stem structure can also be constructed between embryonic V λ and embryonic J λ , although the homology is lower than that of the V κ –J κ stems (Fig. 4d). We propose that the hybrid stem structures are part of the recognition signals for the putative site-specific recombinase.

In all cases known, a few base pairs occur between either one or both cut points of recombination and the stem bases. Thus, for the V–J joining leading to the MOPC321 κ chain gene, the only case in which the exact cut points are known, three base

pairs intervene between the cuts on V21 κ -C and the stem bases, whereas the cuts on J4 are actually at the bases (Fig. 4a). We do not know whether this relationship between the cut points and the stem bases applies to other recombination events between various pairs of V κ s and J κ s, nor whether recombination between V κ 21-C and J4 always occurs at the same sites as shown in Fig. 4a. As residue 96 is a hot spot^{10,11}, one intriguing possibility is that the recombinase can cut and rejoin the DNA strands of a given pair of V and J at slightly different positions so that different triplet codons are reconstituted at the recombination site. For example, the hypothetical recombination events between V21 κ -C and J1 (Fig. 4b) may occur in any one of the following four ways:



where recombinations 1 and 2 give the same dipeptide Pro(95)–Trp(96), whereas recombinations 3 and 4 generate Pro(95)–Arg(96) and Pro(95)–Pro(96), respectively. The latter two recombination events can account for the group III and group IV J peptides, respectively (see Table 1). It follows that the total number of J κ DNA segments might be considerably less than the number of different J κ peptides.

The inverted repeat structures described above have some resemblance to those found at the ends of prokaryotic insertion elements. Ohtsubo and Ohtsubo¹⁴, and Grindley¹⁵ determined sequences of IS1 and found that some 30 bases at one end are invertedly repeated on the other end. Inverted repeat sequences of similar length have also been found at the ends of most insertion elements. Integration of insertion elements into host DNA generates 5- or 9-base pair direct repeats at the outer ends of the inverted repeat sequences^{15–22,36}. The conserved palindromic sequences, CACAGT near V genes and CACTGTG near Js, can be considered to be equivalent to these direct repeats observed at the margins of integrated insertion elements. Although these structural similarities may be coincidental, they could reflect a common evolutionary origin or a common enzymatic mechanism for the two recombination systems, or both.

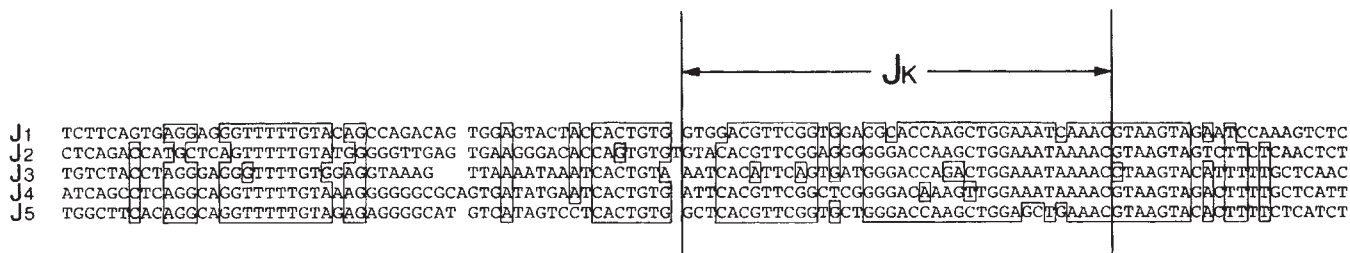


Fig. 3 Comparison of nucleotide sequences of the five J-coding and the flanking regions. Nucleotides common in at least four Js are outlined. Vertical lines indicate determined or predicted ends of the J-coding DNA segments.

V-J joining probably results in deletion

Several models have previously been proposed for recombination of immunoglobulin genes². In the 'copy-insertion' model, a specific V DNA segment is duplicated and the copy is inserted at a site adjacent to a J DNA segment. The 'excision-insertion' model suggests that a specific V DNA segment is excised into an episome-like structure, and this in turn is integrated adjacent to a J DNA segment. According to the 'deletion model' the entire DNA in the interval between a particular pair of V and J DNA segments loops out, is excised, and is diluted out on subsequent cell multiplication. In the 'inversion' model the V and the C DNA (also J DNA) segments are arranged in opposite directions, and a segment of chromosome between a particular V DNA (inclusive) and a J DNA (exclusive) is inverted.

In the context of these models, let us consider the fate of the DNA sequences immediately adjacent to the 3'-end of V, or

immediately adjacent to the 5'-end of J. In all the models except for the deletion model, either one of the two sequences or both should recombine with DNA sequences elsewhere in the genome on V-J joining. In contrast, the deletion model predicts that both sequences should be eliminated. To test these two alternatives, we investigated the λ I chain gene in which both V and J are unique. We digested embryo and myeloma (H2020) DNA with various restriction enzymes, fractionated the digests in a 0.8% agarose gel, and analysed the DNA by Southern gel-blotting procedures. As the hybridisation probes we used a DNA fragment of about 300 base pairs from an embryonic V λ I clone, Ig99 λ , or from an embryonic C λ I clone, Ig25 λ , that includes the respective recombination site as shown in Fig. 5E. Both DNA fragments contain not only the sequence to be examined (*b* or *c*) but also an adjacent sequence (*a* or *d*) known to be incorporated into the rearranged λ chain gene. We deliberately chose such DNA fragments as hybridisation probes to let the *a* and *d* part serve as internal hybridisation controls.

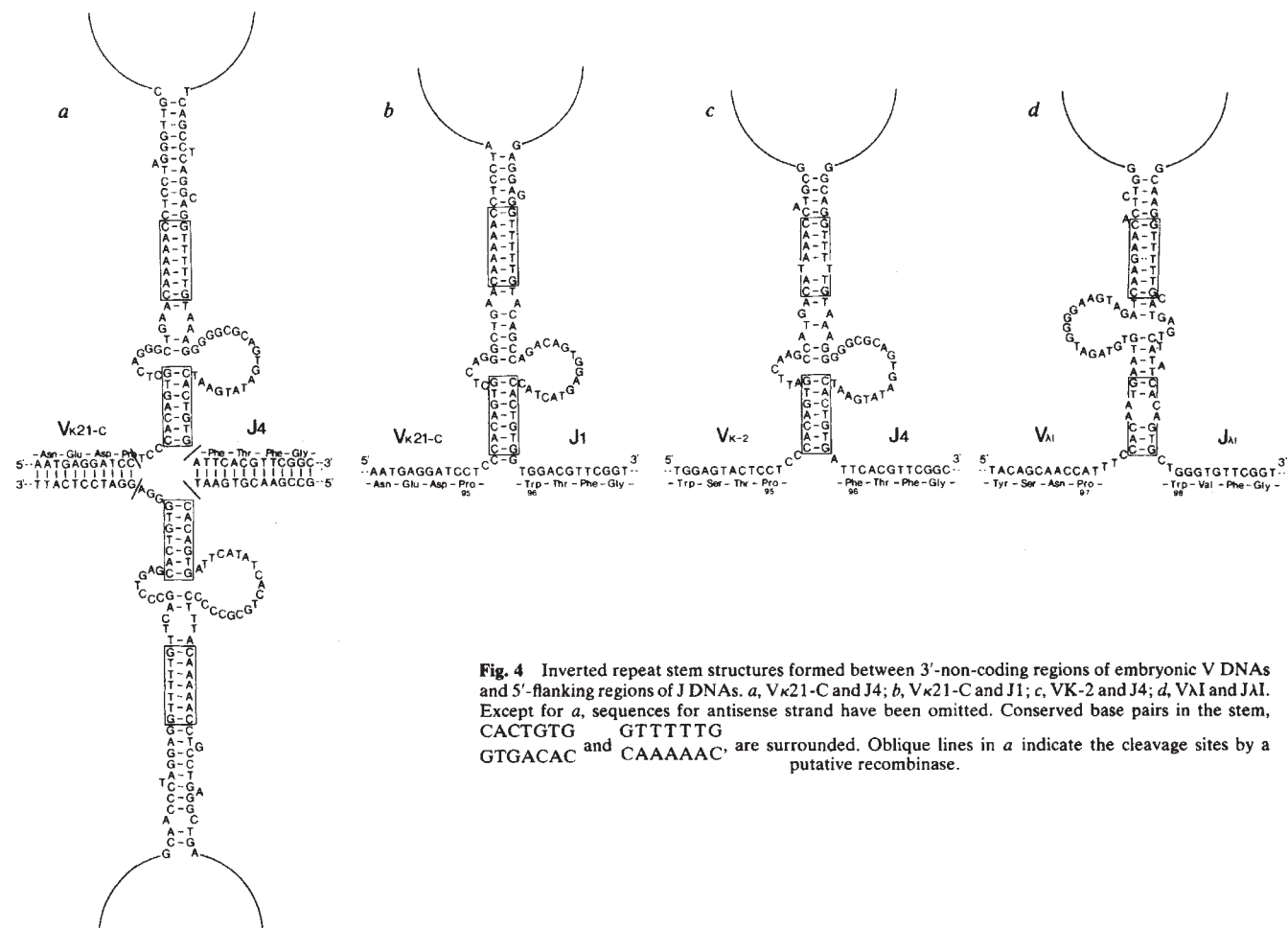


Fig. 4 Inverted repeat stem structures formed between 3'-non-coding regions of embryonic V DNAs and 5'-flanking regions of J DNAs. a, V κ 21-C and J4; b, V κ 21-C and J1; c, V κ -2 and J4; d, V λ I and J λ I. Except for a, sequences for antisense strand have been omitted. Conserved base pairs in the stem, CACTGTG GTTTTG GTGACAC and CAAAAAC are surrounded. Oblique lines in a indicate the cleavage sites by a putative recombinase.

Figure 5A compares hybridisation patterns of embryo and myeloma DNA that were digested with *Eco*RI and analysed with the *ab* probe. Embryo DNA gave two bands of 4.8 and 3.5 kilobases that had previously been identified as λ II- and λ I-containing fragments, respectively³⁻⁵. In the myeloma DNA an additional band of 7.4 kilobases known to contain the rearranged, complete λ I gene⁴ was observed, but no previously unidentified band was present, which would have been expected if the DNA sequence corresponding to the *b* part of the probe had recombined with another DNA sequence. Results of analogous experiments obtained with the *cd* probe are shown in Fig. 5C. Embryo DNA gave a band of 8.6 kilobases known to contain the CAI sequence, and another band, X. The latter band contained a sequence homologous to JAI and its flanking regions (unpublished observations). Myeloma DNA gave these two bands and an additional band of 7.4 kilobases containing the rearranged λ I chain gene³. Again, we observed no myeloma-specific band that could be attributed to the hypothetically recombined DNA containing the *c* part of the probe. Similar results obtained with *Hind*III are shown in Fig. 5B and D. We did not observe myeloma-specific DNA fragments that could not be accounted for by the rearranged, complete λ I chain gene with either probe. We have carried out similar experiments using *Kpn*I, *Bam*HI, *Hind*II and *Hae*III, and obtained results that lead us to the same conclusion (data not shown). Although the results do not unequivocally prove the deletion model (more complicated models in which elements of the other models are combined cannot be ruled out), they suggest strongly that it is the correct one.

Evolution of light-chain genes

We thus suggest that V-J joining is a recombination event accompanied by excision of the DNA sequence between the V and J DNA segments. This DNA sequence, which we call *excison*, carries at its ends characteristic inverted repeat sequences consisting of about 30 bases that could form a stem-loop structure somewhat similar to those of prokaryotic insertion elements. In the light of these findings and the intron-exon structure of immunoglobulin genes we will consider some aspects of the evolution of light-chain genes.

The V and C regions of light chains show a weak sequence homology and are similar in their size, in arrangement of intra-chain disulphide bonds and in three-dimensional structure²³⁻²⁵. Based on these observations it has been generally thought that the DNA segments coding for the two regions arose by duplication of a primordial gene coding for a single homology unit (Fig. 6, step *a*). As we discussed elsewhere this duplication step probably generated a 'dimeric gene' in which the ancestral V- and C-coding DNA segments are separated by a spacer²⁶. When this gene is expressed, the entire DNA is transcribed into a single RNA molecule and the spacer sequence is presumed to have been removed by splicing of the V- and C-coding RNA sequences. We assume that the J DNA segment was an integral part of the ancient V DNA because the J region is homologous to the carboxyl end of the C region³⁵.

The ancestral V DNA, together with some flanking sequences, duplicated, triplicated, and so on under pressure to increase V-region diversity (Fig. 6, step *b*). As splicing is most probably an intramolecular reaction, one requirement of such a 'polymeric gene' would be that the V and C DNA sequences are co-transcribed. In the long RNA transcript every V-coding sequence would have one half-pair of RNA splice sites at its 3'-end (the other half-pair is at the 5'-end of the C-coding sequence), so that a series of mature mRNAs containing different V-coding sequence and the same C-coding sequence could be generated. However, the V-C co-transcription requirement would impose a limit on the size of such a polymeric gene because the transcription unit would become intolerably long as the gene grows. To accommodate 200 V-coding DNA segments, a number estimated for mouse κ chains^{2,8,13,27,28}, the transcript would have to be 100 kilobases long even if no spacer

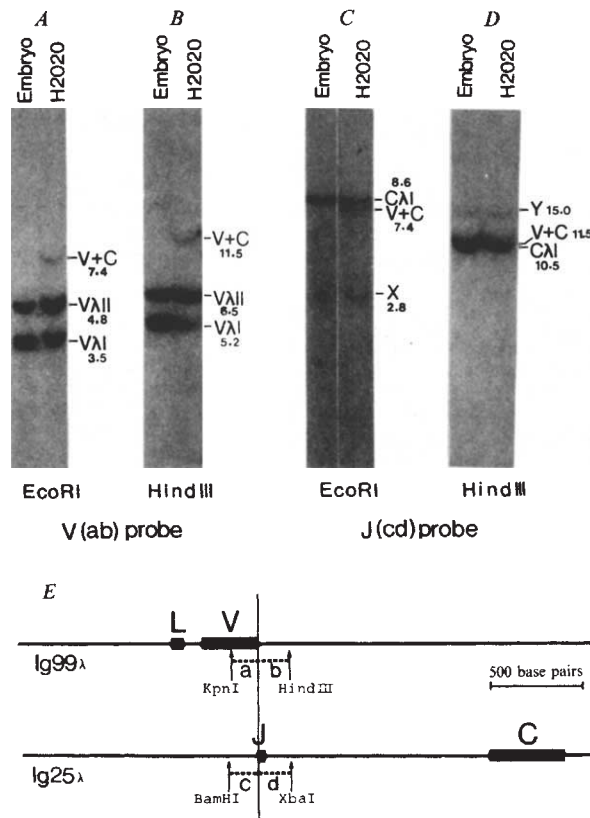


Fig. 5 Southern gel blots of total cellular DNA assayed with hybridisation probes containing the sequences around the recombination sites. High molecular weight DNA extracted from 13-d-old mouse (BALB/c) embryo or from λ I light-chain-producing myeloma, H2020, was digested with *Eco*RI (A and C) or *Hind*III (B and D). The DNA fragments were separated in a 0.8% agarose gel and transferred to a nitrocellulose filter (Schleicher-Schuell, BA85). Filters were then incubated as previously described³ in the presence of 2×10^7 c.p.m. of one of the two nick-translated DNA probes, *a+b* and *c+d*, whose sequence compositions are indicated in E. A and B, hybridisation with the *a+b* probe composed of the 330-base pair *Kpn*I-*Hind*III fragment purified from an embryonic λ I gene clone, Ig99 λ . C and D, hybridisation with the *c+d* probe composed of the 294-base pair *Bam*HI-XbaI fragment purified from an embryonic CAI gene clone, Ig25 λ . Among the bands detected, those indicated to contain λ -coding sequences have previously been characterised by the analysis of DNA clones containing the corresponding DNA fragments. Band X was also cloned, and analysed by electron microscopy of heteroduplexes formed between the cloned DNA and the Ig25 λ DNA. It contains a sequence homologous to JAI and its flanking region (unpublished observation). Band Y has not yet been characterised. Note that both band X and band Y are present not only in the myeloma DNA but also in the embryo DNA. Molecular sizes are indicated in kilobase pairs.

between two adjacent V DNA segments is postulated. The hypothetical transcript may be much larger because at least one such spacer seems to be 10 times longer than a V DNA segment⁹. One obvious solution is to duplicate the entire polymeric gene of a still allowable size. Indeed, all mammals studied so far seem to carry several unlinked sets of light-chain V DNA clusters each associated with a C-coding DNA segment.

An alternative solution is to rearrange DNA sequences somatically so that the V DNA to be expressed is brought in the vicinity of the C DNA in the lymphocytes. We propose that such a mechanism was initiated when an IS-like DNA element was accidentally inserted into one of the multiple V DNA copies of an ancestral polymeric gene, splitting it into two portions, one corresponding to the present day embryonic V DNA segment and the other to the J DNA segment (Fig. 6, step *c*). While this insertion was fixed in the germ-line genome, a mechanism to excise the inserted DNA and rejoin the split V-coding sequences was established in lymphocytes. As excision is a common and major manifestation of prokaryotic IS elements, the basic mechanism for such a process would probably have been

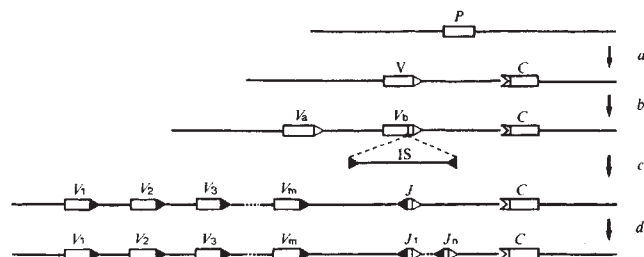


Fig. 6 A hypothetical scheme for the evolution of the κ -light-chain genes. *P*: a primordial gene coding for a single homology unit of immunoglobulin chains. *V*: an ancient variable-region gene. *C*: a constant-region gene. *IS*: a DNA element thought to be inserted into one of the *V* copies, splitting it to a 5'-portion, *V*, and a 3'-portion, *J*. $\triangleright$ and $\triangleleft$ designate a pair of splice sites. $\blacktriangleright$ and $\blacktriangleleft$ designate inverted repeat sequences at the ends of *IS*. Step *a*, duplication of *P* and generation of a pair of splice sites at or near the inward ends of *V* and *C*. Step *b*, limited multiplication of *V* and accidental insertion of *IS* into one of the multiplied *V*s, *V*_b. Step *c*, multiplication of *V* and one half-pair of the inverted repeat sequences. Step *d*, multiplication of *J* and the other half-pair of the inverted repeat sequences. See text for details.

available in vertebrates. Once an ancestral *V* DNA is split, the major body of the *V*-coding sequence and the flanking sequences containing one of the two invertedly repeated sequences can multiply independently of the *J* DNA (Fig. 6, step *c*). Because every copy of the duplicated *V* DNA would have the excision-recombination site at its 3'-end, it can rejoin with the *J* DNA which is kept in the vicinity of the *C* DNA. Because the rearranged *V* DNA can bring in its own promoter, it is no longer necessary to confine the duplicated *V* DNA copies and the *C* DNA within the same transcription unit in the germ-line genome.

Multiplication of *J* DNA segments is an additional aspect of the evolution of light-chain genes (Fig. 6, step *d*). Amino acid sequencing studies of mouse κ chains suggest that a single *J* DNA segment can recombine with more than one *V* DNA segment in different lymphocyte clones. Conversely, a single *V* DNA segment seems to be capable of recombining with more than one *J* DNA segment^{10,11}. In addition, a single *J* DNA segment can give rise to multiple *J* peptides by a slight shift of the recombination site between a given pair of *V* and *J*. Although the pressure under which *J* DNA segments multiplied is unknown, diversification of *V* regions may have been the primary source. As residue 96, the first amino acid fully encoded in *J*, is one of the third complementarity-determining residues²⁹, the *J* peptide could contribute to the antigen-binding activity of the antibody.

Thus, the *V*-*J* joining may well be a reversal of an ancient accidental insertion of an IS-like DNA element which was subsequently exploited by vertebrates to increase the *V*-region diversity within the general framework of the intron-exon structure of higher cell genes.

We thank D. J. McKean and M. Potter for unpublished results, Y. Shimura for restriction enzyme *Ava*II, T. Friedmann for advice on the 3'-end labelling of restriction fragments, T. Knaak for the computer program, G. Dastoornikoo and André Traunecker for technical assistance, and T. Bickle, M. Julius and R. Maki for reading the manuscript.

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- Hozumi, N. & Tonegawa, S. *Proc. natn. Acad. Sci. U.S.A.* **73**, 3628-3632 (1976).
- Tonegawa, S., Hozumi, N., Matthysens, G. & Schuller, R. *Cold Spring Harb. Symp. quant. Biol.* **41**, 877-889 (1976).
- Brack, C., Hiram, M., Schuller, R. & Tonegawa, S. *Cell* **15**, 1-14 (1978).
- Bernard, O., Hozumi, N. & Tonegawa, S. *Cell* **15**, 1133-1144 (1978).
- Tonegawa, S., Maxam, A. M., Tizard, R., Bernard, O. & Gilbert, W. *Proc. natn. Acad. Sci. U.S.A.* **74**, 3171-3175 (1977).
- Klessing, D. F. *Cell* **12**, 9-21 (1977).
- Berget, S. M., Moore, C. & Sharp, P. A. *Proc. natn. Acad. Sci. U.S.A.* **74**, 3171-3175 (1977).
- Lenhard-Schuller, R., Hohn, B., Brack, C., Hiram, M. & Tonegawa, S. *Proc. natn. Acad. Sci. U.S.A.* **74**, 4709-4713 (1978).
- Brack, C. *et al.* (in preparation).
- McKean, D. J., Bell, M. & Potter, M. *Proc. natn. Acad. Sci. U.S.A.* **75**, 3913-3917 (1978).
- Weigert, M., Gatmaitan, L., Loh, E., Schilling, J. & Hood, L. *Nature* **276**, 785-790 (1978).
- Breathnach, R., Benoist, C., O'Hare, K., Cannon, F. & Chambon, P. *Proc. natn. Acad. Sci. U.S.A.* **75**, 4853-4857 (1978).
- Seidman, J. G. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **75**, 3881-3885 (1978).
- Ohtsubo, H. & Ohtsubo, E. *Proc. natn. Acad. Sci. U.S.A.* **75**, 615-619 (1978).
- Grindley, N. D. F. *Cell* **13**, 419-426 (1978).
- Ohtsubo, H., Ohmori, H. & Ohtsubo, E. *Cold Spring Harb. Symp. quant. Biol.* **43** (in the press).
- Calos, M. P., Johnrud, L. & Miller, J. H. *Cell* **13**, 411-418 (1978).
- Allet, B. *Cell* **16**, 123-129 (1979).
- Oka, A., Nomura, N., Sugimoto, K., Sugisaki, H. & Takanami, M. *Nature* **276**, 845-847 (1978).
- Rosenberg, M., Court, D., Shimatake, H., Brady, C. & Wulff, D. L. *Nature* **272**, 414-422 (1978).
- Johnsrud, L., Calos, M. P. & Miller, J. H. *Cell* **15**, 1209-1219 (1978).
- Ghosal, D., Sommer, H. & Saedler, H. *Nucleic Acids Res.* **6**, 1111-1122 (1979).
- Hill, R. L., Delaney, R., Fellow, R. E. & Lebovitz, H. E. *Proc. natn. Acad. Sci. U.S.A.* **63**, 1762-1768 (1966).
- Edelman, G. M. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **63**, 78-85 (1969).
- Poljak, R. J. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **70**, 3305-3310 (1973).
- Sakano, H. *et al.* *Nature* **277**, 627-633 (1979).
- Weigert, M. & Riblet, R. *Cold Spring Harb. Symp. quant. Biol.* **41**, 837-846 (1976).
- Valbuena, O., Marcu, K. B., Weigert, M. & Perry, R. P. *Nature* **276**, 780-784 (1978).
- Wu, T. T. & Kabat, E. A. *J. exp. Med.* **132**, 211-250 (1970).
- Tiemeier, D., Enquist, L. & Leder, P. *Nature* **263**, 526-527 (1976).
- Smith, H. O. & Birnstiel, M. L. *Nucleic Acids Res.* **3**, 2387-2398 (1976).
- Boliver, E. *et al.* *Gene* **2**, 582-564 (1978).
- Maxam, A. & Gilbert, W. *Proc. natn. Acad. Sci. U.S.A.* **74**, 560-564 (1978).
- Klenow, H., Hansen, K. & Patkar, S. A. *Eur. J. Biochem.* **22**, 371-381 (1971).
- Hill, R. L., Belamey, R., Fellows, R. E. & Lavovitz, H. E. *Proc. natn. Acad. Sci. U.S.A.* **56**, 1762-1768 (1966).
- Kleckner, N. *Cell* **16**, 711-720 (1979).

Statistical mechanics of supercoils and the torsional stiffness of the DNA double helix

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The distribution of closed unknotted polymer chains over the writhing number is calculated by the Monte-Carlo method. For circular duplex DNA the variance of the distribution equals approximately half the observed variance of equilibrium distribution over the linking number. The balance which arises from fluctuations in DNA twisting makes it possible to estimate the torsional stiffness of the double helix.

MANY intriguing questions such as the spatial structure of superhelical DNA and the packaging of DNA inside virus particles and chromosomes, are often discussed in terms of the conformational flexibility of the double helix. In most cases changes in the DNA tertiary structure are thought to stem from small continuous local deformations of the double helix¹⁻⁷. Within the framework of this model, DNA can be thought of as an elastic rod with properties completely characterised by its bending and torsional stiffnesses⁸. The resistance of DNA to the bending of the axis of the double helix is deduced from the experimentally determined DNA persistence length using the Bresler and Frenkel theory⁹ (see also refs 10, 11).

Although in the past there has been an ambiguity in the value of the DNA persistence length, recent precise measurements using new techniques have provided a reliable estimate of about 600 Å in standard ionic conditions (0.2 M Na⁺)¹²⁻¹⁶. This has made it possible to calculate the energy associated with the bending of the helix axis in DNA packaging within viruses⁵, nucleosomes⁶ and in the formation of the DNA superhelix⁷.

The value of the torsional stiffness of the double helix, however, remains obscure. This is partly because the torsional stiffness does not affect any properties of linear DNA molecules. On the other hand, all properties of closed duplex DNA should depend in one way or another on the torsional stiffness of the double helix. But in most cases experimentally observed characteristics depend only indirectly on torsional stiffness, and recent attempts to evaluate the torsional stiffness have led to contradictory results^{17,18}.

In this article we demonstrate that the experimental data of Depew and Wang¹⁹ and Pulleyblank *et al.*²⁰ offer a reliable estimation of the DNA torsional stiffness. These authors measured the equilibrium distribution of closed duplex DNA molecules over the number of titratable superhelical turns after enzymatic conversion of open circular (nicked) molecules into the closed form. Although this experimental distribution is determined by thermal fluctuations both in the torsional winding of the double helix and in the number of its tertiary turns^{19,21}, the latter may be theoretically calculated provided that the bending stiffness is known. The balance makes it possible to estimate the torsional stiffness of the double helix.

Rigorous formulation of the problem

Our treatment is based on the important results obtained in the field of differential topology during the last 20 years²²⁻²⁵. Fuller²⁶ was the first to emphasise the crucial significance of these findings for an analysis of closed duplex DNA and overtly introduced the important notion of the writhing number (Wr). This was defined as the difference between the topological characteristic of closed duplex DNA, the linking number (Lk) of the two complementary strands, and the total twist (Tw) of the strands measured along the helix axis.

The concepts of Lk , Tw and Wr are explained at length by Fuller^{26,27} and Crick²⁸. The central point is that the writhing number depends only on the spatial arrangement of the helix axis. Roughly speaking, this concept corresponds to our intuitive notion of the number of tertiary turns. However, even in the simplest case of a regular superhelix, the writhing number does not equal the number of superhelical turns²⁶⁻²⁸.

In terms of the values of Lk , Tw and Wr , the problem of quantitative interpretation of the experimental results on equilibrium closing of nicked DNA^{19,20} may be formulated as follows²¹. In nicked DNA the deviation ΔTw in twisting from the mean value and changes in the writhing number occur due to thermal motion. The experimentally determined value of ΔLk is expressed through these fluctuating characteristics:

$$\Delta Lk = \Delta Tw + Wr \quad (1)$$

It is obvious that the averaged values $\langle Wr \rangle = \langle \Delta Tw \rangle = \langle \Delta Lk \rangle = 0$. However, the values of $\langle (\Delta Tw)^2 \rangle$ and $\langle (Wr)^2 \rangle$ should be nonzero and because in a nicked molecule the fluctuations in twisting and in the number of tertiary turns occur independently from each other^{19,21}:

$$\langle (\Delta Lk)^2 \rangle = \langle (\Delta Tw)^2 \rangle + \langle (Wr)^2 \rangle \quad (2)$$

The value of $\langle (\Delta Lk)^2 \rangle$ was determined experimentally^{19,20,29}. On the other hand, the value of $\langle (Wr)^2 \rangle$ is fully determined by the bending stiffness of the helix axis. So, if it were theoretically calculated using the known value of the DNA bending stiffness, the value of $\langle (\Delta Tw)^2 \rangle$ would remain the only unknown quantity in equation (2). The value of $\langle (\Delta Tw)^2 \rangle$ is connected with the value of the torsional stiffness in a simple way.

So, to determine the DNA torsional stiffness from the experimental data^{19,20,29}, one should calculate the $\langle (Wr)^2 \rangle$ value.

To this end we used the Monte-Carlo method. To obtain a reliable estimate of the desired value the calculation scheme should meet the following requirements: (1) An algorithm should make it possible to generate closed chains in space with a correct probability distribution. (2) A strict algorithm should make it possible to calculate the writhing number for an arbitrary spatial curve. (3) Since the experiments^{19,20,29} were performed with molecules extracted from cells and virus particles in the closed duplex form, the samples most probably contained only unknotted molecules (in a general case the process of replication of a knotted DNA molecule should meet with obstacles³⁰). However, when one randomly generates a closed chain it may prove to be knotted with some definite probability³⁰. To discard such knotted chains in the course of the Monte-Carlo calculations a reliable computer algorithm is needed to recognise them. (4) Since the Monte-Carlo calculations are performed for some simplified models of the real DNA molecule, one should prove that the results are still valid when one passes to more realistic models.

Note that Benham²¹ who recently attempted to solve the same problem used a calculation scheme that satisfied none of the above requirements. In contrast, the calculation scheme outlined below meets all the above requirements and makes it possible to obtain, for the first time, a reliable estimation of the $\langle (Wr)^2 \rangle$ value.

The calculation scheme

To calculate the writhing number for a closed curve in space simulating the position of the central axis of a DNA molecule we have used two independent but equally rigorous approaches.

The first one is as follows²⁶. A curve is projected onto a plane perpendicular to an arbitrary direction. For this projection the so-called directional writhing number^{21,26} is calculated. The writhing number Wr is the average of the directional writhing

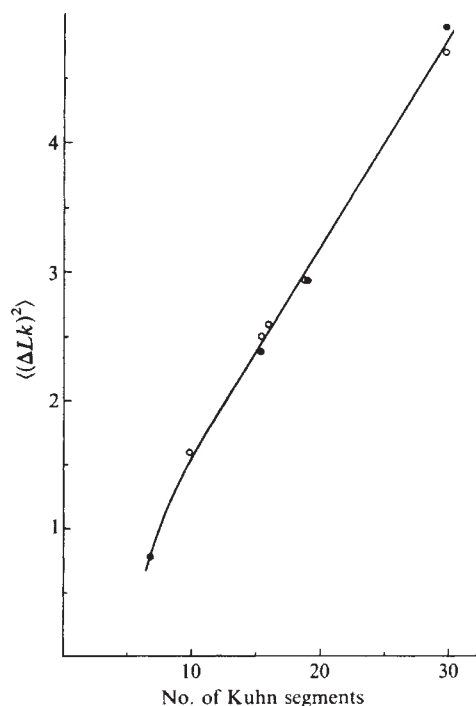


Fig. 1 Experimental dependence of the variance of equilibrium distribution over the linking number Lk on the number of Kuhn statistical segments in DNA molecules. The points correspond to the following DNAs (the values in parentheses are the numbers of base pairs): plasmid 15 of *E. coli* (2,300); minicol (3,300); SV40 (5,224); polyoma (5,380); Col E1 (6,360); fd (6,408); PM2 (10,050). The $\langle (\Delta Lk)^2 \rangle$ values were taken from refs 20, 29 (○) and ref. 19 (●). The Kuhn statistical length was chosen to be equal to 1,150 Å (338 base pairs)¹⁶.

number over all directions. A practical limitation of this method is that to obtain a reliable Wr value one has to analyse a great number of projections of a curve in different directions, so this is too time-consuming for routine Monte-Carlo calculations.

Another method based on a representation of the writhing number in terms of the gaussian integral is more convenient. It follows from the definition of the Wr value²⁶ and from the results in refs 22–25 that

$$Wr = \frac{1}{4\pi} \iint \frac{(\mathbf{dr}_1 \times \mathbf{dr}_2) \cdot \mathbf{r}_{12}}{r_{12}^3} \quad (3)$$

where $\mathbf{r}_1$ and $\mathbf{r}_2$ are the radius vectors whose ends run along the curve and $\mathbf{r}_{12} = \mathbf{r}_1 - \mathbf{r}_2$. Although in routine calculations we used the second method based on equation (3), our main results were also verified by the first method. Note that when the first method is used in Monte-Carlo calculations it is essential that the directional writhing number for each generated curve be averaged over its different projections before averaging the value $\langle (Wr)^2 \rangle$ over the different curves. Otherwise $\langle (Wr)^2 \rangle$ values are overestimated (this is one of Benham's²¹ faults). A great advantage of the use of equation (3) in the Monte-Carlo calculations is that in these calculations the curves always consist of straight segments. We therefore represented the double integral in equation (3) for a total curve as the double sum over these segments and used an analytical expression for the value of the integral for a pair of straight segments.

To see if a randomly generated closed curve is knotted, we have calculated the topological invariant of knot (Alexander polynomial) as we did previously in our study on statistical mechanics of knots^{30,31}.

The Monte-Carlo calculations can be performed only for some simplified polymer models. These calculations are simplest for a model of freely joined straight segments, but actual DNA is most probably a continuous worm-like structure. It is known, however, that various mean characteristics of a polymer chain depend on the number of Kuhn statistical segments alone, and are independent of the particular polymer model, if the number of Kuhn segments is not too small. Special calculations show that this is also true for $\langle (Wr)^2 \rangle$. Indeed, the results hold as one passes from the free-joint model to a model with a free rotation of adjacent segments within a cone determined by a fixed angle between the segments. We compared the results obtained on the two models for the same number of Kuhn statistical segments. So we believe that our results should be valid for the worm-like model which is a limit of the model with free rotation.

We did not take into consideration the excluded volume effects because they are known to be negligible in ordinary conditions for the DNA chain lengths involved.

Finally, note that there is a very effective algorithm for the model of a freely joined chain (A. M. Dykhne, unpublished), making it possible to generate only closed chains in space. There is no such algorithm for the free-rotation model. To check the validity of our results for the free-rotation model we repeated some of the calculations using a very cumbersome method involving straightforward generation of arbitrary chains of a given length with a subsequent selection of those having a small enough distance between the ends.

Results

Figure 1 shows all the published experimental data on the variance of equilibrium distribution over the linking number $\langle (\Delta Lk)^2 \rangle$ as a function of DNA chain length. Chain length is expressed in terms of the number of Kuhn statistical segments, using the Kuhn statistical length of 1,150 Å given by Kovacic and van Holde¹⁶. Note that other recent estimations of this quantity^{12–15} differ insignificantly from the chosen value (the Kuhn statistical length is double the persistence length). In the original papers^{19,20} it was proved that the experimentally measured distribution over the linking number was in fact an equilibrium distribution. Note the high accuracy of the experimental data, as demonstrated by the very good agreement

between results obtained in two different laboratories with several different DNA preparations and with two different enzyme systems (DNA ligase and a nicking-closing enzyme).

Figure 2 shows the results of our calculations of the variance of distribution over the writhing number $\langle (Wr)^2 \rangle$ as a function of the number of Kuhn segments in a closed polymer chain. The dotted line shows the results obtained when the $\langle (Wr)^2 \rangle$ value was calculated for all randomly generated closed chains both unknotted and knotted. The solid line corresponds to the results obtained for unknotted chains only. As mentioned above, it is probable that only the latter results correspond to the experimental situation. Note that although for the number of segments under study the percentage of knotted chains is very small (not more than about 5%, see refs 30, 31) they contribute appreciably to the $\langle (Wr)^2 \rangle$ value. Thus analysis of randomly generated chains on their topological state proved to be absolutely essential (see also below).

Figures 1 and 2 show that the calculated value of $\langle (Wr)^2 \rangle$ is almost equal to half the experimental $\langle (\Delta Lk)^2 \rangle$ value. Our data sharply contradict the data of Benham²¹ who calculated a value for $\langle (Wr)^2 \rangle$ which was about four times larger than ours. In this connection it should be stressed that our calculations meet all the above-listed requirements and their statistical error is negligible. Our results can therefore be used for obtaining, from the experimental data summarised in Fig. 1, a reliable estimation of the variance of the twisting of DNA strands about the central axis $\langle (\Delta Tw)^2 \rangle$ which, according to equation (2), is simply the difference $\langle (\Delta Lk)^2 \rangle - \langle (Wr)^2 \rangle$. Figure 3 shows the results.

The result presented in Fig. 3 was obtained for a particular Kuhn statistical length (1,150 Å). The accuracy of the latter may be estimated at about 10% (see refs 12–16). It means that the possible error in the $\langle (\Delta Tw)^2 \rangle$ value estimation is about 20%.

The ΔLk value has a gaussian distribution^{19,20,29}. Our calculations show that the same is true of the Wr value. It follows from these data and equation (1) that the ΔTw value should be normally distributed.

Let us now interpret the obtained value of $\langle (\Delta Tw)^2 \rangle$ in terms of the DNA torsional stiffness. We shall only consider the simplest model of independent variations of adjacent angles between nucleotide pairs. If a DNA molecule consists of $N + 1$ base pairs it has N such independent angles. For each of them

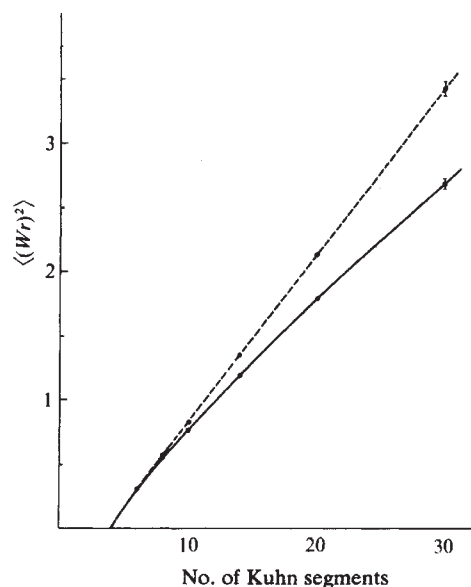


Fig. 2 Results of calculations of the variance of distribution over the writhing number Wr for a closed polymer chain as a function of the number of Kuhn statistical segments in it. The solid line refers to the case of unknotted chains only. The dotted line corresponds to the case when all randomly generated closed chains, both unknotted and knotted, are taken into consideration. The error bar is shown for the case of 30 segments. In all other cases the statistical error of the Monte-Carlo calculations is negligible.

one can use a parabolic dependence of the energy f on the deviation $\Delta\tau$ of the angle from its equilibrium position:

$$f = \frac{1}{2}g(\Delta\tau)^2 \quad (4)$$

where g is the value of torsional stiffness.

The probability of a given value of $\Delta\tau$ should conform to the Boltzmann equation

$$P(\Delta\tau) \sim \exp\left(-\frac{f}{RT}\right) = \exp\left(-\frac{g(\Delta\tau)^2}{2RT}\right) \quad (5)$$

As a result the variation in the total twisting angle of the molecule expressed in terms of the number of revolutions will have a gaussian distribution with the variance

$$\langle(\Delta Tw)^2\rangle = N \frac{RT}{g} \quad (6)$$

So, the value of $\langle(\Delta Tw)^2\rangle$ should be directly proportional to N and the slope of the straight line determines the value of torsional stiffness g . In accordance with equation (6) the calculated value of $\langle(\Delta Tw)^2\rangle$ is directly proportional to N (see solid line in Fig. 3). This result is by no means trivial and is valid only for precise calculations. This is demonstrated by the dotted line in Fig. 3, which was obtained from the same experimental data as Fig. 1, but using the theoretical curve for the $\langle(Wr)^2\rangle$ value calculated without the exclusive selection of unknotted chains (the dotted line in Fig. 2).

If the $\Delta\tau$ value is measured in angular degrees, then the numerical value of torsional stiffness which follows from the application of equation (6) to the data of Fig. 3, is

$$g = 0.036 RT \quad (7)$$

It follows from the above estimation of the accuracy of determination of the $\langle(\Delta Tw)^2\rangle$ value that the obtained g value should be accurate to within 20%.

Note that we have restricted ourselves to considering only the simplest model of DNA torsional fluctuations. In principle, the same value of $\langle(\Delta Tw)^2\rangle$ may originate, for instance, from rare but marked changes in the winding angle of the double helix. An ultimate choice between different models cannot be made on the basis of the obtained $\langle(\Delta Tw)^2\rangle$ value alone. As mentioned in the introductory section, the above model which treats DNA as an elastic rod seems today preferable to other models. Note that there is no contradiction between this model and the concept of local fluctuational opening of the double helix^{32,33}, because the probability of the latter process in linear or nicked DNA is too small to account for the observed bending as well as torsional flexibility.

Discussion

The above results make it possible to clear up several intriguing questions. First the data lead directly to the conclusion that half of the total number of the titratable superhelical turns in closed duplex DNA is realised as tertiary turns (writhing number) and the other half is realised as the axial twisting (or untwisting) of the double helix. Note that all experimental techniques give the number of titratable superhelical turns only (see for example ref. 34). Our results make it possible for the first time to infer the number of tertiary turns (more exactly, the writhing number) from the experimentally determined number of titratable superhelical turns which is of great importance, for example, for a quantitative interpretation of the hydrodynamic properties of superhelical DNAs. The number of titratable superhelical turns remains strictly constant under fixed external conditions in a given molecule, but this constant value may be distributed in different ways between writhing and twisting. Using the results of theoretical treatment of the problem by Le Bret¹⁷ and our results it is clear that the distributions over the writhing number and twisting in closed duplex DNA are virtually identical and their variance amounts to half the variance in nicked DNA calculated above.

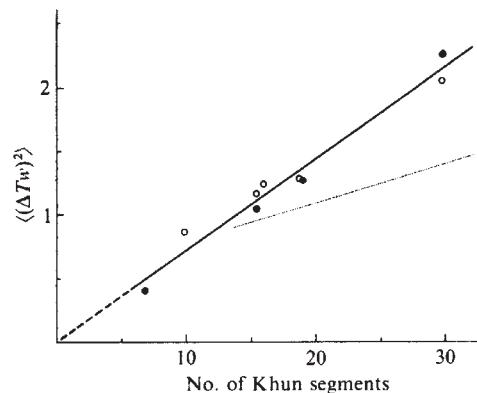


Fig. 3 The variance of the distribution over axial twisting Tw , calculated on the basis of Figs 1 and 2. Points are obtained from the experimental points in Fig. 1 and the solid line in Fig. 2 which corresponds to unknotted chains. The straight solid line is plotted through the resulting points. The dashed line is obtained from the same experimental data as for Fig. 1 but using the dotted line in Fig. 2 which was obtained without exclusive selection of unknotted chains.

For example, let us consider PM2 DNA. It has, in standard conditions, about 100 titratable superhelical turns³⁴. So, the number of tertiary turns (the writhing number) for this DNA should be about 50, with the root-mean-square deviation of about unity. Note that the latter value does not contradict the fact that a sample containing the superhelical DNA molecules with a given number of topological turns gives a very sharp band in gel electrophoresis with a halfwidth much smaller than the distance between two bands which differ by one topological turn (that corresponds to only half a tertiary turn). As was emphasised by Depew and Wang¹⁹, the width of a single band does not reflect the distribution of molecules with a given topology over different numbers of tertiary turns. So this width cannot be used for estimating the variance of the distribution as Le Bret¹⁷ attempted to do.

Our estimation of the DNA torsional stiffness makes it possible to evaluate, for the first time, the free energy that is required to change the DNA winding angle at a given value. This free energy proved to be unexpectedly small. For instance, a change in the angle of 2.6° , which is the difference in this angle between the B and C forms of DNA, corresponds to a change in free energy of no more than $0.12 RT$ (72 cal mol^{-1} of base pairs).

Assuming that angles between different base pairs change independently one can estimate the amplitude (root-mean-square deviation) of thermal fluctuations in the value of the angle between adjacent base pairs that proves to be as large as $\pm 5^\circ$. This may be directly estimated from the data in Fig. 3 without any additional assumptions (the amplitude is equal to $(\langle(\Delta Tw)^2\rangle/N)^{1/2}$). Note that, within the framework of the continuous model, this value may be overestimated only if there is a strong correlation between changes in the winding angle of sites far removed from each other. Of course, some correlation may exist but it is not very likely to be significant.

The large fluctuations in the winding angle that follow from our results most probably reflect more complex fluctuational changes in the DNA conformation. Theoretical conformational analysis suggests that the energy map of DNA has a long 'ravine' that corresponds to forms of the so-called B family³⁵. It is natural to assume that the fluctuations in the winding angle correspond to a motion along the bottom of this ravine. In this case one can expect that along with the large fluctuations in the winding angle, other parameters, such as the inclination of base pairs (tilt) and the propeller of bases in the pairs should also markedly fluctuate. Presumably it is these fluctuations that lead to the considerable absorption of the light polarised along the helix axis³⁶. Note that our concept does not contradict the results

of X-ray analysis of DNA fibres just because these fluctuations in fibres may be much smaller than those seen in solution due to intermolecular interaction.

Our conclusion that the large variability in DNA structural parameters can entail simple thermal motion may help clarify the mechanism of DNA-protein recognition. Indeed, these data throw great doubt on any concept that relates that recognition to small variations of DNA structure in sites with different sequences.

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1. Namoradze, N. Z., Goryunov, A. N. & Birshtein, T. M. *Biophys. Chem.* **7**, 59–70 (1977).
2. Sussman, J. L. & Trifonov, E. N. *Proc. natn. Acad. Sci. U.S.A.* **75**, 103–107 (1978).
3. Levitt, M. *Proc. natn. Acad. Sci. U.S.A.* **75**, 640–644 (1978).
4. Zhurkin, V. B., Lysov, Y. P. & Ivanov, V. I. *Nucleic Acids Res.* **6**, 1081–1096 (1979).
5. Riemer, S. C. & Bloomfield, V. A. *Biopolymers* **17**, 785–794 (1978).
6. Camerini-Otero, R. & Felsenfeld, G. *Nucleic Acids Res.* **4**, 1159–1181 (1977).
7. Camerini-Otero, R. & Felsenfeld, G. *Proc. natn. Acad. Sci. U.S.A.* **75**, 1708–1712 (1978).
8. Landau, L. D. & Lifshitz, E. M. *Theory of Elasticity* (Pergamon, London, 1959).
9. Bresler, S. E. & Frenkel, Y. I. *Zh. Eksp. Teor. Fiz. USSR* **9**, 1094–1106 (1939).
10. Landau, L. D. & Lifshitz, E. M. *Statistical Physics* (Addison-Wesley, Reading, Mass., 1958).
11. Schellman, J. A. *Biopolymers* **13**, 217–226 (1974).
12. Yamakawa, B. & Fujii, M. *Macromolecules* **7**, 125–135; 649–654 (1974).

13. Record, M. T. Jr., Woodbury, C. P. & Inman, R. B. *Biopolymers* **14**, 393–408 (1975).
14. Godfrey, J. E. & Eisenberg, H. *Biophys. Chem.* **5**, 301–318 (1976).
15. Jolly, D. & Eisenberg, H. *Biopolymers* **15**, 61–95 (1976).
16. Kovacic, R. T. & van Holde, K. E. *Biochemistry* **16**, 1490–1498 (1976).
17. Le Bret, M. *Biopolymers* **17**, 1939–1955 (1978).
18. Belintsev, B. N., Gagua, A. V. & Nedospasov, S. A. *Nucleic Acids Res.* **6**, 983–992 (1979).
19. Depew, R. E. & Wang, J. C. *Proc. natn. Acad. Sci. U.S.A.* **72**, 4275–4279 (1975).
20. Pulleyblank, D. F., Shure, M., Tang, D., Vinograd, J. & Vosberg, H.-P. *Proc. natn. Acad. Sci. U.S.A.* **72**, 4280–4284 (1975).
21. Benham, C. J. *J. molec. Biol.* **123**, 361–370 (1978).
22. Calugareanu, G. *Rev. Math. pure appl.* **4**, 5–20 (1959).
23. Calugareanu, G. *Czech. Math. J.* **11**, 588–625 (1961).
24. Pohl, W. F. *Am. J. Math.* **90**, 1321–1345 (1968).
25. White, J. H. *Am. J. Math.* **91**, 693–728 (1969).
26. Fuller, F. B. *Proc. natn. Acad. Sci. U.S.A.* **68**, 815–819 (1971).
27. Fuller, F. B. *Proc. natn. Acad. Sci. U.S.A.* **75**, 3557–3561 (1978).
28. Crick, F. H. C. *Proc. natn. Acad. Sci. U.S.A.* **73**, 2639–2643 (1976).
29. Shure, M., Pulleyblank, D. E. & Vinograd, J. *Nucleic Acids Res.* **4**, 1183–1205 (1977).
30. Frank-Kamenetskii, M. D., Lukashin, A. V. & Vologodskii, A. V. *Nature* **258**, 398–402 (1975).
31. Vologodskii, A. V., Lukashin, A. V., Frank-Kamenetskii, M. D. & Anshelevich, V. V. *Sov. Phys.-JETP* **39**, 1059–1063 (1974).
32. Frank-Kamenetskii, M. D. & Lazurkin, Y. S. A. *Rev. Biophys. Bioengng* **3**, 127–150 (1974).
33. Lukashin, A. V., Vologodskii, A. V., Frank-Kamenetskii, M. D. & Lyubchenko, Y. L. *J. molec. Biol.* **108**, 665–682 (1976).
34. Bauer, W. A. *Rev. Biophys. Bioengng* **7**, 287–313 (1978).
35. Zhurkin, V. B., Lysov, Y. P. & Ivanov, V. I. *Biopolymers* **17**, 377–412 (1978).
36. Hogan, M., Dattagupta, N. & Crothers, D. M. *Proc. natn. Acad. Sci. U.S.A.* **75**, 195–199 (1978).

letters

Weak low-latitude X-ray sources and local galactic structure

A LONG-STANDING problem of astronomy has been the elucidation of conditions that prevail in the local solar neighbourhood. Starting with Gould¹, work has continued to try to map out the morphology of the Milky Way in the vicinity of the Sun. It was noted a century ago¹ that the nearby OB stars were not distributed symmetrically with respect to the galactic plane. We report here some of the interesting contributions that X-ray sources make towards this picture.

Evidence is mounting that the low latitude ($|b^{\text{II}}| < 20^\circ$), weak ($S < 200$ Uhuru c.p.s.) X-ray sources are situated in the solar neighbourhood. I have shown previously that the latitudinal distribution of these sources places them probably within ~ 2 kpc of the Sun, radiating between 10^{32} and 10^{34} erg s⁻¹ in the 2–6 keV range². This is corroborated by the discovery of stars such as Algol and γ Cas radiating in this energy band with this luminosity, as well as by models of Fabian, Pringle and Rees³ that yield X-ray energies of this magnitude originating from accretion onto white dwarfs.

A remarkable statistic emerges when the galactic latitude of these sources ($5 < S < 50$ c.p.s.) is plotted from the 4U catalog⁴: 32 of the sources have negative galactic latitudes whereas only eight have positive values. The probability that this is a chance occurrence of a uniformly distributed population is 9×10^{-5} , calculated by

$$\sum_{i=S}^n \binom{n}{i} \times 0.5^n,$$

where $S = 32$ and $n = 40$. Thus, this is a statistically significant ($\approx 4\sigma$) indication of a non-random distribution of X-ray sources about the galactic plane. Furthermore, if one extends the analysis to 200 c.p.s. (where confusion will be caused by galactic centre sources), and to 4 c.p.s. (where confusion will be caused by extragalactic sources), the statistic remains $> 3\sigma$. For the inclusive population ($4 < S < 200$ c.p.s.), 50 sources have negative latitudes and 25 are positive. This is shown in Fig. 1, and

summarised in Table 1. Note that the Ariel 5 satellite identified 22 new sources not in the Uhuru catalog: 15 are below the plane, and seven are above^{5,6}. Unfortunately, the statistics are not adequate for a two-parameter fit to an equation of the type $b^{\text{II}} = A \sin l^{\text{II}} + B \cos l^{\text{II}}$ (see ref. 2), but a coincidence emerges when gross features are examined. For example, in the region $90^\circ < l^{\text{II}} < 300^\circ$, 22 sources ($4 < S < 200$) have negative galactic latitudes, and six are positive. This is close to the region where most local O and B stars are found to be below the galactic plane^{7–9}, and is further evidence that the weak sources reside in the solar neighbourhood. In fact, within 300 pc of the Sun, 115 O and B stars are located below the plane, and 79 are above⁹, an asymmetry which is approximately 3σ . This result is subject to observational selection as it excludes parts of the southern Milky Way; thus, the asymmetry will, if anything, be enhanced.

Extending the analogy with local objects we can for the first time understand the $\log N \log S$ slope of -0.4 , discovered by Matilsky, *et al.*¹⁰ and later verified by Forman *et al.*⁴ using the improved data from the 4 Uhuru catalog. As pointed out by Johnson¹¹, the -0.4 slope does not arise simply from any spiral arm geometry. However, all previous analyses assumed a uniform density distribution, and this is probably not the case. Because of the unique position of the Sun in a locally dense

Table 1 Distribution of X-ray sources with $4 < S < 200$ c.p.s.

Uhuru intensity	No. of sources	Latitude interval	Probability
$5 < S < 50$	$\begin{cases} 32 \\ 8 \end{cases}$	$b^{\text{II}} < 0^\circ$ > 0	9×10^{-5}
$5 < S < 200$	$\begin{cases} 38 \\ 17 \end{cases}$	< 0 > 0	3×10^{-3}
$4 < S < 200$	$\begin{cases} 50 \\ 25 \end{cases}$	< 0 > 0	2.6×10^{-3}
$4 < S < 50$	$\begin{cases} 44 \\ 16 \end{cases}$	< 0 > 0	2×10^{-4}

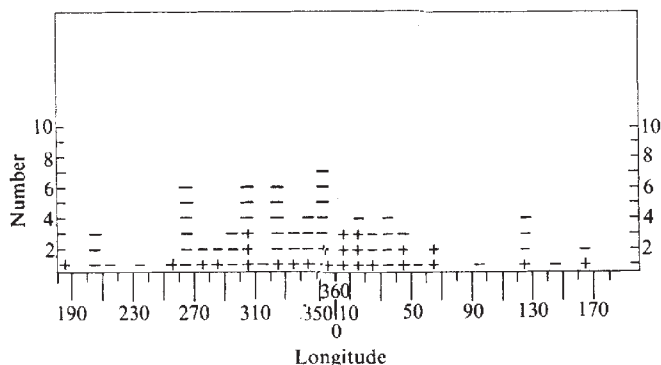


Fig. 1 Distribution of $|b| < 20^\circ$ sources with $4 < S < 200$ Uhuru c.p.s. Number of sources in 10° longitude bins is plotted. '+', the source lies above the plane; '-', negative galactic latitude. Supernova remnants and clusters of galaxies are excluded.

region of a spiral arm, the density function of B stars decreases with increasing distance from the solar neighbourhood, in the region from 300 pc to ~2,000 pc. Although there are subtle variations with galactic longitude due to obscuration and other effects, McCuskey⁸ has derived composite density functions which indicate an approximate density $D_s(r) \propto 1/r$ for early B stars. Thus it does not seem inappropriate to assume that a similar function exists for the local X-ray sources. This does not, of course, mean that these X-ray sources are members of typical OB star binary systems but merely that these sources probably have a local distribution function similar to that delineated by the nearby, early type stars. These may be single early type stars whose X-ray emission is due to a stellar wind interacting with the ambient interstellar medium¹², although the X-ray flux ought to be a bit softer than that of a Uhuru source. It is more probable, however, that these objects are similar to Wray 977 (4U1223-62), a 40 c.p.s. source, identified with a $m_v = 11$, B1 supergiant, which has a long (21 d) period, and whose smaller X-ray flux is due to the decreased mass transfer associated with the wide separation of the components. To arrive at a -0.4 slope for $d \log N/d \log S$, $D_s(r)$ will be proportional to $r^{-1.2}$, for the galactic sources distributed in the disk. There are, of course, possibilities that source confusion and source variability can obscure this straightforward view. However, these effects do not seem to change substantially our picture of the extragalactic component¹³, which generally contains yet fainter sources and is represented by a steeper $\log N/\log S$ slope, which thereby ought to cause more severe problems than the galactic distribution. Thus it seems that a negative source density gradient in the solar neighbourhood is the most probable explanation of the distribution of the low luminosity X-ray sources.

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- Gould, B. A. *Uranometria Argentina*, 355 (1879).
- Matilsky, T. *Astrophys. J. Lett.* **217**, L83 (1977).
- Fabian, A. C., Pringle, J. E. & Rees, M. J. *Mon. Not. R. astr. Soc.* **175**, 43 (1976).
- Forman, W. et al. *Astrophys. J. Suppl.*, **38**, 357 (1978).
- Seward, F. D. et al. *Mon. Not. R. astr. Soc.* **175**, 39P (1976).
- Seward, F. D. et al. *Mon. Not. R. astr. Soc.* **177**, 13P (1976).
- Blaauw, A. *Astrophys. J.* **123**, 408 (1956).
- McCuskey, S. W. *Astrophys. J.* **123**, 458 (1956).
- Lesh, J. R. *Astrophys. J. Suppl.* **17**, 371 (1968).
- Matilsky, T. et al. *Astrophys. J.* **181**, 753 (1973).
- Johnson, H. M. *Astrophys. J.* **224**, 381 (1978).
- Castor, J. I., McCray, R. & Weaver, R. *Astrophys. J. Lett.* **200**, L107 (1975).
- Murray, S. preprint Center for Astrophysics.

Differential solar rotation depends on solar activity

SUNSPOTS have long been used as tracers to determine the rotation rate of the Sun. Scheiner¹ noted 250 years ago that low-latitude sunspots rotate more rapidly than those at high latitude ('differential rotation'). In a recent review, Howard² stressed the limitations of the sunspot technique for determining rotation rate and differential rotation, as long-lived spots may have substantial individual motions, random and systematic, in both longitude and latitude. For example, leading and following spots of a sunspot group diverge early in the group's lifetime³; rotation rates for isolated ('unipolar') spots and groups differ, with group rates depending on the shape of the group⁴. Another problem in using sunspots is the Wilson effect, a surface depression of the spot causing errors in longitude estimates away from the central meridian. Despite these difficulties, spots have been used previously to establish that there is no secular variation in rotation rate and differential rotation, nor any variations with phase of the solar cycle⁵, the only possible exception being during long-term disruptions in the solar activity cycle mechanism⁶. We present here evidence to suggest that the first of these conclusions is incorrect, and that differential rotation does depend on the amplitude of the solar cycle, at least for unipolar spots.

The time-averaged differential rotation law for sunspots may be written as

$$\langle \omega \rangle = \alpha + \beta \sin^2 \phi + \gamma \sin^4 \phi \quad (1)$$

where $\langle \omega \rangle$ gives the daily angular sidereal motion in terms of solar latitude ϕ . Since sunspots are concentrated to within 30° latitude of the solar equator, the γ term is usually ignored. The most quoted determination for $\langle \omega \rangle$ is that of Newton and Nunn⁵, who found from an analysis of sunspot data for the interval 1934 to 1944 that

$$\langle \omega \rangle = 14.38 (\pm 0.01) - 2.96 (\pm 0.09) \sin^2 \phi \text{ deg per day} \quad (2)$$

Newton and Nunn used stable recurrent spots in their determination: 174 unipolar spots dominated their analysis, although 89 leader and 8 follower spots of groups were also included. Because of what is known about non-uniform characteristic motions, particularly in groups, Newton and Nunn's comparison of the 1934-44 data with information on earlier cycles (given in refs 7 and 8) may be invalid. Data selection for the earlier cycles seems to have been based on the longevity of spots, irrespective of whether they were unipolar or leaders of groups—the actual fraction of each is not given, although reference is made to 'the large percentage of cases' where spots used were group leaders. Thus the evidence for the long-term invariance of differential rotation is inadequately founded. However Newton and Nunn's result for the invariance during a single cycle apparently stands, despite the small data sample near cycle minimum.

Since Ward³ found that rotation estimates were a function of spot size and shape, with 'streamers' moving more quickly than 'bipolar' spots, which in turn move faster than unipolar spots, for comparison of daily angular sidereal motion from cycle to cycle it is necessary that only unipolar spots of restricted size range be used. We have done this for three years centred near the maxima of the solar cycles which peaked in 1883-85 and 1956-58, at the extremities of the 80-y 'Gleissberg Cycle'. The annual mean sunspot number at the maximum of the 11-y cycle, $R_{\max}$, for 1956-58 was three times that for 1883-85, $R_{\max}$ having increased steadily between the two. Spot sizes were limited to 30 to 300 millionths of the visible solar disk, and latitudes to less than 30° . Although spot positions are available in the 'Daily Results' and 'Ledgers' of the Royal Observatory (based on plates taken at Greenwich, and subsequently Herstmonceux, and complemented with plates from Dehra Dûn, Mauritius, Cape of Good Hope, Kodaikanal, and Mt Wilson), all plates and

prints for the two epochs were individually checked and where necessary spot positions remeasured. An additional selection criterion was spot longevity (at least 10 days). The only relaxation of these conditions was that the short-term appearance of a small companion to a long-lived unipolar spot was ignored. Spot positions within 10° of the limb were not used because of difficulties in determining accurate longitude from a foreshortened image of the spot. A more stringent criterion is needed for estimating the centre-of-gravity for sunspot groups; however we found that for unipolar spots positional estimates were reproducible and accurately determinable beyond 10° of the limb.

The strict application of the above selection criteria severely restricted the data sample to only 1 to 2 spots per month—50 long-lived unipolar spots were found suitable for 1883–85, and 67 for 1956–58. (Extending the time intervals to include spots from near cycle minima would not have significantly increased the data sample size because of the scarcity of long-lived unipolar spots near cycle minima.) The latitude ranges for the data used (combining northern and southern hemispheres, since there is no apparent difference in spot rotation rates, ref. 5) were—

Latitude	No. of spots	
	1883–85	1956–58
0–5°	6	3
5–10°	17	8
10–15°	13	17
15–20°	5	11
20–25°	7	20
25–30°	2	8

A total of 354 spot positions for 1883–85 and 525 positions for 1956–58 were included in the subsequent analysis. A least-squares analysis was used to determine the sunspot rotation relationship for $\phi \leq 30^\circ$.

1883–85 $R_{\max} \sim 60$

$$\langle \omega \rangle = 14.372 (\pm 0.012) - 2.628 (\pm 0.166) \sin^2 \phi \quad \text{deg per day} \quad (3)$$

1956–58 $R_{\max} \sim 180$

$$\langle \omega \rangle = 14.349 (\pm 0.011) - 3.367 (\pm 0.094) \sin^2 \phi \quad \text{deg per day} \quad (4)$$

In this analysis it was assumed that the spots had a common proper motion in both longitude and latitude, and it was further assumed that the motion in latitude is directed towards the equator. Individual proper motions of the spots were ignored.

It seems that the equatorial rotation rate, α , remained essentially constant over the interval of interest, a result in accord with previous investigations (for example, refs 1–5) when standard errors are accounted for. However, Eddy (personal communication) has detected a variation in equatorial rotation rate between 1890 and 1960 by fitting all Greenwich sunspot group data to a modified rotation law

$$\langle \omega \rangle = a + b\phi + c\phi^2$$

and allowing for spot proper motions fitted to a quadratic. (b lies within the experimental uncertainty of value zero, and may be ignored.) By fitting our data to this same relationship, we confirm our initial finding of constant ' a ' for the 1883–85 and 1956–58 maxima. Indeed, only the 1890–1900 cycle in the Eddy analysis shows a value markedly discrepant with our conclusion of constant equatorial rotation rate.

There seems to be a dramatic variation in differential rotation factor β , in the sense that high-latitude spots rotate more slowly with increasing $R_{\max}$. Expression (2) is compatible with such an interpretation, since $R_{\max}$ for the 1938–39 maximum was ~ 110 , although it must be stressed that the Newton and Nunn results did include a significant fraction (about one third) of leader and follower spots. A 20% higher estimate for our ' c ' value for the 1956–58 maximum compared with Eddy's is compatible with Ward's conclusion regarding the differing behaviour of groups and unipolar spots.

All the above determinations depend on the adopted value of i , the inclination of the solar equator to the ecliptic, and Ω the longitude of the ascending node of the solar equator on the ecliptic. The values used today were estimated by Carrington⁹ over a century ago. Recently Wöhl¹¹ has pointed out that there could be large errors in Carrington's values for i and Ω . The constant of differential rotation β would change if i and Ω were changed. Carrington used the latitude observations of individual sunspots covering an 8-yr interval to derive the constants. Since we have similar data we attempted to re-evaluate the solar elements and the results are shown together with estimates of the internal errors in the table below.

	Present evaluation	Carrington (1863)
i	7.22 ± 0.02	7.25
Ω_{1850}	73.94 ± 0.20	73.67

The present value of Ω is $\Omega_{1850} + 0.01396t^\circ$ where t is the number of years since 1850. The error in Ω is larger than in i because the method enables the quantities $\Omega \sin i$ and i to be determined, and their errors are about equal. These results are in the same sense but much smaller than those predicted by Wöhl¹¹ who gives a table of previous determinations from sunspots and calculates his own set of elements from Doppler measurements of the solar plasma, which differs considerably from Carrington's values.

In view of the possibility of large systematic errors in the estimation of Carrington's elements, and the small difference in these new values it seems advisable to continue to use Carrington's values published in 1863 until techniques are further improved.

A satisfactory explanation of differential rotation remains a matter of debate—for a recent review see ref. 10. The evidence presented here suggests that any future theory must account for an invariant equatorial rotation rate α , but a differential factor β which varies with the amplitude of the sunspot cycle, at least in the case of unipolar spots.

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1. Scheiner, C. *Rosa ursina sive sol* (Andreas Phaeus, Braccuab, Italy, 1630).
2. Howard, R. *Rev. Geophys Space Phys.* **16**, 721 (1978).
3. *Mon. Not. R. astr. Soc.* **85**, 553 (1925).
4. Ward, F. *Astrophys. J.* **145**, 416 (1966).
5. Newton, H. W. & Nunn, M. L. *Mon. Not. R. astr. Soc.* **111**, 413 (1951).
6. Eddy, J. A., Gilman, P. A. & Trotter, D. E. *Solar Phys.* **46**, 3 (1976).
7. *Mon. Not. R. astr. Soc.* **85**, 548 (1925).
8. Newton, H. W. *Mon. Not. R. astr. Soc.* **95**, 60 (1935).
9. Carrington, R. C. *Observations of the Spots on the Sun* (Williams and Norgate, London, 1863).
10. Gillman, P. A. in *Basic Mechanisms of Solar Activity*, IAU Symp. 71 (eds Bumba, V. & Kleczek, J.), 207 (Reidel, Dordrecht, 1976).
11. Wöhl, H. *Astr. Astrophys.* **62**, 165 (1978).

Origin of the jovian ring and the galilean satellites

THE discovery by Voyager I of a thin flat stream of rocks orbiting Jupiter at a radius of some 130,000 km, or $\sim 2R_p$ ($R_p = 6.677 \times 10^9$ cm = present polar radius), in its equatorial plane opens a new avenue of information in our understanding of the formation of the Solar System. Each of the major planets which possesses a regular satellite system also possesses a ring system. Theories for the formation of the major planets should

therefore address themselves to this phenomenon of the co-appearance of rings and regular satellite systems, especially in considering the question whether Neptune, which has no regular satellites, may also not have a ring. We suggest here that Jupiter's rocky belt formed through condensation from a gaseous disk which was shed by the rotating gaseous envelope that originally contracted to form that planet. The four galilean satellites were formed from gaseous rings which were shed by the same envelope at an earlier and more turbulent stage in its evolution. It is possible that Jupiter may possess another rocky satellite belt at orbital radius $4R_P$.

The regular satellite systems of Jupiter, Saturn and Uranus are remarkable for the near coplanarity and circularity of their orbits. In addition, the orbital radii R_n , when numbered from the outermost members inwards ($n = 0, 1, 2, \dots$), form nearly perfect geometric sequences

$$R_n/R_{n+1} \approx \text{constant} = \Gamma \quad (1)$$

where for Jupiter $\Gamma = 1.65$. We have recently² suggested that these regular satellite systems were formed in a manner similar to the hypothesis originally advanced by Laplace³ for the formation of the planetary system; namely, through condensation from a set of gaseous rings which were shed by the primitive contracting gaseous envelopes, which make up the bulk of the mass of these planets^{4,5}. Now, although Laplace's hypothesis was largely abandoned over a century ago because of a number of objections^{6,7}, one of us (A.J.R.P.) has shown these objections can be overcome if one takes into account a supersonic turbulent stress arising from the motions of overshooting convective elements within the cloud^{8,9}.

The turbulent stress has a radial component given by $\langle \rho_i v_i^2 \rangle = \beta \rho GM(r)/r$ where ρ is the gas density and $M(r)$ the mass interior to radius r , while β is the so-called turbulence parameter^{10,11}. Typically, for $\beta \approx 0.1$ the turbulent stress is some 30 times the normal gas pressure $\rho RT/\mu$ just beneath the photosurface of the cloud. The main effect of this stress is to cause the gaseous envelope to become very centrally condensed; that is, it drastically lowers the moment of inertia $I \equiv f M_{\text{env}} R_e^2$, where M_{env} is the total envelope mass, and R_e is the equatorial radius. Typically, we find $f \approx 0.01$ – 0.02 . The turbulent stress also leads to the development of a very dense shell of non-turbulent gas at the surface of the cloud. As the cloud contracts to the fully rotating configuration at radius R_0 , given where the centrifugal force at the equator first matches the gravitational force there, the dense outer shell evolves into a belt-like structure at the equator. This belt, of mass m , is left behind by the cloud while the latter withdraws dynamically to a smaller radius, stabilising itself rotationally through the extrusion of fresh material to the surface. The gas density of the detached ring diminishes with distance ξ from the mean Kepler orbit R_0 according to

$$\rho(\xi) = \rho_0 \exp(-\frac{1}{2} \alpha \xi^2 / R_0^2) \quad (2)$$

where $\rho_0 = \alpha m / 4\pi^2 R_0^3$ is the density on the mean orbit and $\alpha = \mu GM_{\text{env}} / RT_0 R_0 = \mathcal{O}(10^2)$.

The process of shedding gas rings repeats itself at the sequence of orbital radii R_n which satisfy the equations

$$R_n/R_{n+1} = [1 + m/M_{\text{env}} f]^2 \quad (3)$$

Thus if the contraction occurs homologously, meaning that both m/M_{env} and f are preserved, then R_n/R_{n+1} is constant and the sequence R_n is geometric. Next, taking $R_n/R_{n+1} = 1.65$ and $f = 0.02$, equation (3) tells us that the masses of the jovian rings were each about the same and equal to

$$m = 1.1 \times 10^{28} \text{ g}$$

Such a mass of gas of solar composition contains rock and rock + H₂O ice components

$$m_{\text{rock}} \approx 5 \times 10^{25} \text{ g}, \quad m_{\text{rock} + \text{H}_2\text{O ice}} \approx 12 \times 10^{25} \text{ g}$$

The observed masses of the rocky satellites Io and Europa are 9×10^{25} and 5×10^{25} g, respectively, whilst the ice-like satellites Ganymede and Callisto have masses 15×10^{25} and 11×10^{25} g. This suggests that the protojovian envelope probably did shed rings of gas of roughly equal mass equal to that shown above.

To determine the chemistry of the galilean satellites, a turbulent polytropic numerical model of the fully rotating protojovian envelope was constructed having mass $M_{\text{env}} = M_J = 1.901 \times 10^{30}$ g. The turbulence parameter $\beta = 0.109$ is chosen so that the Titius-Bode 'constant' $\Gamma = \Gamma(\beta) = 1.65$. During uniform contraction the ratio of surface to central temperatures T_s/T_c is a fixed fraction θ_{ph} of the ratio of surface to central molecular weights μ_s/μ_c . Choosing $\theta_{\text{ph}} = 0.003$ we achieve a model where the condensation point for H₂O ice lies between the orbits of Europa and Ganymede, as required by the observational data.

The results of the calculations are summarised in Table 1. There we show the envelope moment-of-inertia coefficient f , the ratio P_s of turbulent stress to gas pressure just beneath the photosurface, and the temperature T_n and density ρ_n on the mean orbit of each gas ring just after the moment of detachment t_n from the planetary envelope, which is measured from initial radius $R_e = 30 R_P$. We assume that a gas ring was shed at the orbit of each of the satellites and that Callisto's orbit marks the radius R_0 where the contracting envelope first became rotationally unstable. The cloud is energetically stable at each radius because of the turbulent support. The factor f increases slowly to 0.015 during the contraction owing to the steady dissociation of H₂. The final column details the composition of the condensate at each orbit, derived from the chemical condensation sequences computed by Lewis¹².

We note that Io and Europa form from a mixture of hydrous mineral silicates. For Io, the Fe combines with S to form troilite, whilst for Europa the Fe appears as FeO. As the various condensates draw together along the mean orbit of the gas ring to form the satellite, the heat released through their compaction

Table 1 Physical properties of the protojovian envelope and gas rings and the expected composition of the galilean satellites

Satellite	Orbital radius R_n/R_P	Moment-of-inertia coefficient f	Turbulent stress ratio P_s	Temperature T_n (K)	Gas density ρ_n (g cm ⁻³)	Time of ring detachment t_n (10 ⁶ yr)	Chemical composition of the condensate
Io	6.3	0.0150	26.1	533	4.4×10^{-4}	14.2	CaTiO ₃ , MgSiO ₃ , Ni, (Na, K)AlSi ₃ O ₈ , FeS, tremolite, talc
Europa	10.1	0.0148	25.9	338	1.1×10^{-4}	11.4	As above, with FeS → FeO (= 'rock')
Ganymede	16.0	0.0141	24.9	220	2.4×10^{-5}	7.3	rock, H ₂ O ice
Callisto	28.2	0.0130	23.5	133	4.0×10^{-6}	0.8	rock, H ₂ O ice

will cause the S as well as H_2O to leach out from the growing satellite's interior. This may account for the copious deposits of S observed on the surface of Io, as well as for the icy plains of Europa. For Europa, the black-body temperature due to the jovian envelope falls below the H_2O ice point some 2.5×10^7 yr after its formation, long after its gaseous ring has evaporated. The satellites Ganymede and Callisto condense below the H_2O ice point in this model and therefore consist of dirty ice.

According to the present theory, the contracting envelope also shed one or two more gaseous rings at orbital radii about $4R_p$ and $2.5R_p$. Why then do there appear to be no major satellites at these interior positions? The answer to this question lies in the rate of settling of the condensate particles compared with the dissipation rate of the gaseous ring. The gaseous rings thermally evaporate over a time scale $t_{\text{evap}} \sim 10^4$ yr. The condensing particles settle onto the central keplerian orbit R_n of the gas ring over a time $t_{\text{set}} \propto 1/\rho_s a^2$ where, at Io's orbit, $t_{\text{set}} = 10^3$ yr for particles of radius $a = 10^{-2}$ cm and mass density $\rho_s = 3 \text{ g cm}^{-3}$. The galilean satellites are therefore expected to contain their full share of condensate. Inside Io, however, where the temperature is higher ($T_n \propto R_n^{-1}$), the particles are less hydrous and more dusty, and therefore more likely to remain suspended in the gas as a fine smoke¹³. In addition, the inner rings are most likely stirred by the increased envelope luminosity, which may also frustrate the settling out process. The mass of segregated rocky material at orbital radii $4R_p$ and $2.5R_p$ may therefore be only a small fraction of the total mass of condensate (about 5×10^{25} g) which existed at each of these orbits. If, further, this settled mass is less than $\sim 2 \times 10^{18}$ g in the case of a protojovian envelope having $f = 0.015$, or less than 2×10^{21} g for $f = 0.02$, there will be insufficient time for the rocks to aggregate into a single body before the gaseous component of the ring disperses, taking with it the finer material. These mass bounds are consistent with the fact that so far no satellites have been detected at these orbits.

It is possible that the tiny satellite Amalthea (mass $\geq 10^{21}$ g, orbital radius $\sim 2.72 R_p$) somehow formed from the ring near $2.5R_p$. But the dark red spectral features of this body¹⁴, which suggest a composition similar to C-type asteroidal material, together with the absence of any corresponding satellite at $4R_p$, raises instead the more likely possibility that Amalthea is a stray asteroid captured by Jupiter at some time after its formation.

The process of shedding gas rings cannot proceed indefinitely as a uniform contraction of the outer layers of the cloud would lead to unrealistically high surface temperatures T_s . Instead we suggest, following the non-turbulent cloud calculations of Graboske *et al.*¹⁵, that soon after radius $R_c = 3R_p$ has been passed, where $T_s = 1,120$ K, T_s begins to downturn and level off to ~ 600 K by radius $1.5R_p$. Now, a downturn in T_s implies a slowing down in the rate of release of gravitational energy $d\Omega/dt \propto R_c^2 T_s^4$ and hence a diminution in the degree of supersonic turbulent convection in the cloud. But such a drop in turbulence also leads to a closer spacing between the rings of gas, as a weakly turbulent cloud is unable to support as much material at the equator. Computationally, we find that as T_s falls from 1,120 to 600 K, that β falls from 0.11 to 0.07 and $\Gamma = R_n/R_{n+1}$ drops sharply from 1.65 to 1.02 during the contraction from radius $R_c = 3R_p$ to $1.5R_p$, for a cloud which maintains the same $f = 0.015$. For $R_c \leq 2.5 R_p$ the rings are so closely spaced that they essentially merge together to form a continuous disk. Any largish grains condensing out in this dense ($\rho \sim 3 \times 10^{-3} \text{ g cm}^{-3}$) gaseous keplerian disk settle onto the mid-plane to form a rocky sheet. Owing to the vigorous thermal stirring likely to be present in the gas, however, the mass of this solid disk is again likely to be much less than the total available condensate mass $\sim 2 \times 10^{25}$ g. The disk is unable to coalesce into a few single bodies owing to its differential rotation^{16,17}. After the gaseous component of the disk has dispersed, orbital resonances and other tidal phenomena¹⁸ lead to evolutionary changes in its structure, which may account for the observed sharp inner edge at radius $1.9R_p$.

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1. Prentice, A. J. R. *Proc. astr. Soc. Austr.* **3**, 172-173 (1977).
2. Prentice, A. J. R. & ter Haar, D. *The Moon and the Planets* (in the press).
3. Laplace, P. S. *Exposition du Système du Monde*, 387-397 (Courcier, Paris, 1796).
4. Prentice, A. J. R. in *In the Beginning* (ed. Wild, J. P.) 15-47 (Australian Academy of Science, Canberra, 1974).
5. Stevenson, D. J. in *The Origin of the Solar System* (ed. Dermott, S. F.) 395-432 (Wiley, London, 1978).
6. Babinet, J. C. r. *hebd. Séanc. Acad. Sci., Paris* **52**, 481-484 (1861).
7. Maxwell, J. C. in *The Scientific Papers of James Clerk Maxwell Vol. 1* (ed. Niven, W. D.) 288-376 (Cambridge University Press, 1890).
8. Prentice, A. J. R. in *The Origin of the Solar System* (ed. Dermott, S. F.) 111-162 (Wiley, London, 1978).
9. Prentice, A. J. R. *The Moon and the Planets* **19**, 341-398 (1978).
10. Prentice, A. J. R. *Astr. Astrophys.* **27**, 237-248 (1973).
11. Prentice, A. J. R. *Astr. Astrophys.* **50**, 59-70 (1976).
12. Lewis, J. S. *Icarus* **16**, 241-252 (1972); *Earth planet. Sci. Lett.* **15**, 286-290 (1972).
13. Hoyle, F. & Wickramasinghe, N. C. *Nature* **217**, 415-418 (1968).
14. Millis R. L. *Icarus* **33**, 319-321 (1978).
15. Graboske, H. C., Pollock, J. B., Grossman, A. S., & Olness, R. J. *Astrophys. J.* **199**, 265-281 (1975).
16. Toomre, A. *Astrophys. J.* **139**, 1217-1238 (1964).
17. Goldreich, P., & Ward, W. R. *Astrophys. J.* **183**, 1051-1061 (1973).
18. Dermott, S. F., & Gold, T. *Nature* **267**, 590-593 (1977).

Optical absorption coefficients of water

THE absorption spectrum of water in the visible region has been widely investigated¹⁻⁹ because of the basic, technological and environmental importance of liquid water. Despite these efforts, there are significant disagreements between experimental results: discrepancies of factors of ~ 2 in the absorption coefficients are not uncommon between various data. Possible reasons for the disagreements among the various studies are: (1) a lack of a reliable, sensitive technique for measuring small absorption coefficients in liquids; (2) the presence of a significant amount of light scattering by particles (note that the amount of Rayleigh scattering by pure water is quite small and predictable in the visible⁸); and (3) measurements are often not done for pure distilled water stored in a noncontaminating vessel. We present here the first accurate measurement of the absorption coefficient of pure water at 21 °C in the 450-700-nm region. We have utilised a recently developed opto-acoustic (OA) technique¹⁰, using pulsed dye lasers and immersed piezoelectric transducers. This technique is ideally suitable for measuring weak linear or nonlinear absorptions in nonfluorescing neat liquids or solutions^{11,12}. Our present absorption spectra for water, having typical accuracies of $\pm 5\%$, should be useful not only for a basic understanding of the properties of water, but also for practical applications like underwater light propagation or intracavity dye laser measurement of dissolved materials in aqueous solutions.

We used the OA cell shown in Fig. 1. There are several important changes to the cell previously used for benzene¹⁰. We have designed a special transducer with a stainless steel casing which has a flat polished inside surface against which a lead zirconate titanate (PZT) cylinder is pressed. (Details of the cell's construction and its operation will be published later.) This transducer is superior to the commercially available transducers

which we previously used because the possibility of contamination of the water sample and the formation of small air bubbles on the transducer surface are eliminated. The OA cell is filled with triple-distilled deionised water. Since the coefficient of thermal expansion of water changes drastically with temperature¹³, a small chromel–alumel thermocouple in contact with the water is used to monitor the water temperature which is stabilised at $21.5 \pm 0.2^\circ\text{C}$ for the present experiments. This is necessary since the OA signal arising from the absorption of the optical radiation depends crucially on the coefficient of thermal expansion of the liquid as seen from equation (3) of ref. 10. An expansion tube is attached to the side of the OA cell to ensure that the water sample is at atmospheric pressure. With the above precautions, no contamination problems were encountered, and the OA spectrum was reproducible for a freshly distilled water sample as well as for the same sample stored in the OA cell for several weeks. To obtain the absorption spectrum, a flashlamp-pumped dye laser that can scan through the visible region (with various laser dyes) was used. The OA signal amplitude, A , is detected by the submersed transducer, and the laser pulse energy, E , is detected by a pyroelectric detector as the laser frequency, ν , is scanned by a stepping motor drive. A minicomputer collects the data and plots A/E as we scan ν . The normalised OA spectrum is directly proportional to the actual absorption spectrum¹⁰ (note that conventional spectrophotometry measures absorption plus scattering, while here we only measure absorption). The proportionality constant can be obtained by a comparison method^{11,12}; that is comparing the OA signal for pure water with the OA signal for an aqueous solution (water doped with a known small amount of nonfluorescing dye). In the present experiment, KMnO_4 is used as the nonfluorescing dye¹⁴. Absolute absorption coefficient at 659.9 nm is measured to be $(3.7 \pm 0.3) \times 10^{-3}\text{ cm}^{-1}$.

The OA absorption spectrum of distilled water at 21.5°C is shown in Fig. 2, which exhibits a minimum at $21,050\text{ cm}^{-1}$ (475 nm) with a minimum value of $\alpha(21,050) = 1.7 \times 10^{-4}\text{ cm}^{-1}$. It also exhibits distinct shoulders at $19,460\text{ cm}^{-1}$ and at $16,550\text{ cm}^{-1}$. These shoulders, which are absent in heavy water³, are due to sixth and fifth harmonics of the O–H stretch, respectively (being analogous to the absorption peaks observed in liquid benzene¹⁵ due to the harmonics of the C–H stretch). We found that the n th harmonic absorption peak (or shoulder) in water is reasonably well given by the following simple anharmonic formula

$$\nu_n = n(3,620 - 63n)\text{ cm}^{-1} \quad (1)$$

The comparison of experimental peak or shoulder positions in water with the predicted position by equation (1) is given in Table 1.

A comparison of our OA absorption spectrum with attenuation of absorption spectra given by other authors is given in Fig. 2. Of special interest is a comparison with the most recent data of Querry *et al.*⁹ who performed a split laser pulse attenuation

Table 1 Harmonics of the O–H stretch in liquid water. ν_n (cm^{-1}) in the position of an absorption peak ($n = 1-4$) or a shoulder ($n = 5, 6$) for the n th harmonic

n	$\nu_n\text{ cm}^{-1}$ (experimental)	$\nu_n\text{ cm}^{-1}$ (predicted by equation (1))
1	3,401*	3,557
2	6,944*	6,988
3	10,309*	10,293
4	13,441*	13,472
5	16,550†	16,525
6	19,460†	19,452

* See for example ref. 6.

† Present work.

measurement on deionised filtered water. The spectral features exhibited by our data and the data of Querry *et al.* are strikingly similar, except that their attenuation coefficient is displaced upward by about $2 \times 10^{-4}\text{ cm}^{-1}$ from our absorption coefficient. Our data agree reasonably well with the review compilation of Irvine and Pollack⁵, and of Hale and Querry⁶ in the blue region ($450-500\text{ nm}$) but agreements at some points outside this region are sometimes poor. Agreements with Hulbert's² absorption data and Hass and Davisson's⁸ adiabatic laser calorimetry measurements for distilled water in the blue are also good; however, their absorption coefficients are significantly lower than our values at longer wavelengths. The critical compilation of Kopelevich⁷ seems strongly to underestimate the absorption in the $400-500\text{-nm}$ range.

In conclusion, we believe we have reported the first reliable absorption spectrum of pure distilled water at 21.5°C . The absorption spectrum does not change appreciably from 20 to 30°C . An overall comparison of the data by various authors indicates a broad absorption minimum near 475 nm . However, some values differ by as much as an order of magnitude. Agreement on the red side of the 600-nm region is much better and the existing data agree with our measurements to $\pm 20\%$. Our presently reported data are believed to be the most reliable values so far. There are no clear-cut explanations for the large difference among the previous measurements themselves and for the differences between the previous measurements and our present ones. However, we suggest the following problems with the earlier optical transmission studies which measure extinction coefficients. (1) Conventional optical transmission measurements for pure water require long path lengths because of small absorption coefficients. To extract an absorption coefficient from such a transmission measurement, attenuation caused by scattering must be taken into account. Light scattering by suspended small particles can often be significant compared with the true absorption in the blue region. Accurate accounting of such light scattering effect is difficult. (2) Corrections are necessary for absorption and reflection losses at the optical windows. (3) Possible lateral displacements of the optical beam by water sample causes systematic errors. (4) Long cells may often require use of materials which may add contaminants to the pure water. The previous transmission measurements typically require corrections from (1) and (3) above and some authors probably over-correct their data while others under-correct their data. None of these problems limit the reliability of the measurements reported here since the OA technique measures true absorption directly and the careful construction of the OA cell out of primarily stainless steel has very nearly eliminated contamination problems.

The only possible source of error in our measurements exists in the fundamental requirement for quantitative OA spectroscopy that the optical absorption must only result in nonradiative decay producing heat (but no fluorescence or chemical changes). The above requirement is met in our present OA measurements of water and is justified on two considerations: first, vibrational relaxation of polyatomic molecules in liquid state at room temperature is known to proceed on a

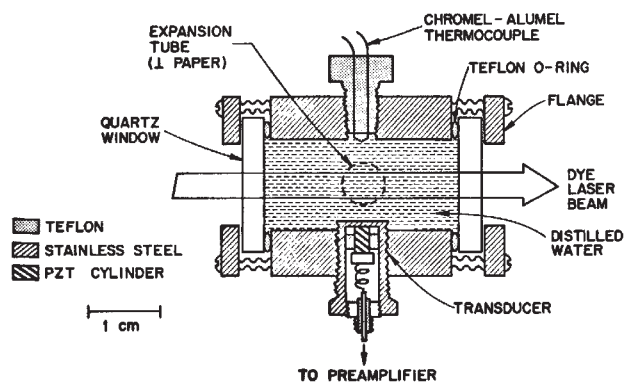


Fig. 1 Experimental OA cell for distilled water.

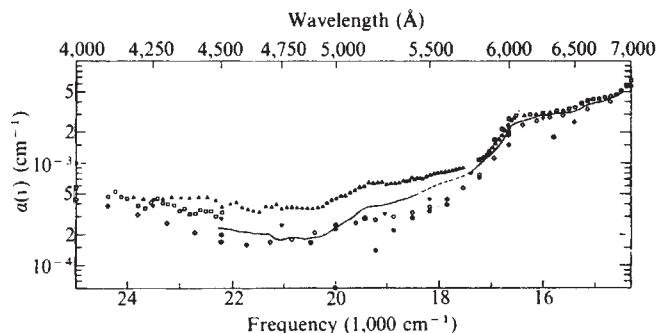


Fig. 2 Absorption spectrum of triple distilled deionised water by OA determination (solid line). The dashed parts are interpolations not covered by the tuning range of the laser dyes used. Also shown are experimental data of other authors for comparison: $\diamond$, ref. 2; $\square$, ref. 3; $\blacklozenge$, ref. 5; ∇ , ref. 6; $\bullet$, ref. 7; $\otimes$, ref. 8; $\triangle$, ref. 9. Statistical accuracy of our data is about $\pm 5\%$. The uncertainty in the absolute calibration (which does not affect the shape of the curve) does not exceed $\pm 10\%$.

picosecond time scale¹⁶. Molecules excited to high vibrational level (high harmonics) are expected to relax even faster as exemplified in the case of benzene¹⁷. Second, fluorescence yield is negligible unless there is a substantial Frank–London overlap between the singlet ground state and the singlet excited electronic state. The lowest known excited electronic state of H_2O is a triplet (3A_2) situated at^{18,19} about $36,300\text{ cm}^{-1}$ (275 nm). Thus the observed absorption in the visible region ($\lambda > 400\text{ nm}$) does not involve electronic excitation. Thus no excited electronic states of H_2O which could fluoresce to the ground state are produced in the present experiment. No fluorescence was experimentally observed in the water samples during our studies. Prominent spectral features are due to harmonics of the O–H stretch and seem to be represented reasonably well by the simple anharmonic formula given in equation (1). No other fine structures in the spectrum (which we measured in high resolution) are found, in agreement with Drummeter and Knestrick.⁴ Detailed tabulation of the absorption coefficients of light and heavy water as well as further details of the comparison between existing data and our new measurements will be given elsewhere²⁰.

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- Clarke, G. L. & James, H. R. *J. opt. Soc. Am.* **29**, 43–55 (1939).
- Hulburt, E. O., *J. opt. Soc. Am.* **35**, 698–705 (1945).
- Sullivan, S. A., *J. opt. Soc. Am.* **53**, 962–968 (1963).
- Drummeter, L. F. & Knestrick, G. L., *Appl. Opt.* **6**, 2101–2103 (1967).
- Irvine, W. M. & Pollack, J. B., *Icarus* **8**, 324–360 (1968).
- Hale, G. M. & Querry, M. R., *Appl. Opt.* **12**, 555–563 (1973).
- Kopelevich, O. V., *Opt. Spectrosc.* **41**, 391–392 (1976).
- Hass, M. & Davison, J. W. *J. opt. Soc. Am.* **67**, 622–624 (1977).
- Querry, M. R., Cary, P. A. & Waring, R. C., *Appl. Opt.* **17**, 3587–3592 (1978).
- Patel, C. K. N. & Tam, A. C., *Appl. Phys. Lett.* **34**, 467–470 (1979).
- Tam, A. C., Patel, C. K. N. & Kerl, R. J., *Opt. Lett.* **4**, 81–83 (1979).
- Patel, C. K. N. & Tam, A. C., *Chem. Phys. Lett.* **62**, 511 (1979); *Nature* **280**, 304–306 (1979).
- Kell, G. S. *J. chem. Engng Data* **12**, 66–69 (1967).
- Lahmann, W. & Ludewig, H. J., *Chem. Phys. Lett.* **45**, 177–179 (1977).
- Patel, C. K. N., Tam, A. C. & Kerl, R. J., *J. chem. Phys.* (in the press).
- Laubereau, A., von der Lide, O. & Kaiser, W., *Phys. Rev. Lett.* **28**, 1162 (1972); *Opt. Commun.* **11**, 74 (1979).
- Reddy, K. V., Bray, R. G. & Berry, J. in *Advances in Laser Chemistry* (ed. Zewail, A.) 48 (Springer, Berlin, 1978).
- Knoop, F. W. E., Brongersma, H. H. & Oosterhoff, L. H., *Chem. Phys. Lett.* **13**, 20 (1972).
- Larzul, H., Gelebart, F. & Johannin-Gilles, A., *C. r. hebdo. Séanc. Acad. Sci. Paris* **261**, 4701 (1968).
- Tam, A. C. & Patel, C. K. N. (in preparation).

Two-photon absorption spectra and cross-section measurements in liquids

THE recently developed high-sensitivity opto-acoustic (OA) absorption measurement technique¹ has proved ideal for the measurement of a variety of weak absorption phenomena in liquids^{2–4}. We report here the results of an investigation of two-photon absorption in liquid benzene using the OA technique. Weak two-photon spectra (cross-section $\leq 10^{-51}\text{ cm}^4\text{ molecule}^{-1}\text{ photon}^{-1}\text{ s}$) in nonfluorescing liquids have been difficult to measure. The only previous published study for such a measurement used thermal blooming⁵, which is difficult to interpret theoretically and apparently produces a poor signal-to-noise ratio. We demonstrate here that far superior spectra can be obtained by the simple and sensitive two-photon OA spectroscopy (TPOAS) technique. Polarisation dependence, vibrational intervals and electronic origin are obtained, and two-photon absorption cross-sections are derived for the first time in this study for the case of a weak two-photon absorption band in liquid benzene. Note that Bonch-Bruевич *et al.*⁶ have recently attempted similar two-photon OA measurements, but they can only measure strong absorption (cross-section $\geq 10^{-48}\text{ cm}^4\text{ molecule}^{-1}\text{ s}$) with a poor signal-to-noise ratio.

The two-photon absorption we study here is the $B_{2u} \leftarrow A_{1g}$ transition of benzene⁷ which is symmetry forbidden but becomes allowed in two-photon absorption due to vibronic coupling. The $B_{2u} \leftarrow A_{1g}$ transitions have been extensively studied in vapour phase benzene^{8–14} by fluorescence monitoring. In the liquid phase, however, fluorescence studies are severely hampered by the rapid nonradiative singlet to triplet relaxation^{15,16} ($> 4 \times 10^7\text{ s}^{-1}$ in liquid benzene at room temperature). Quantum fluorescence yield¹⁵ of the singlet excited state is small (< 0.07) and decreases rapidly with vibrational energy^{9,10}. The triplet state produced also undergoes rapid nonradiative decay^{17–19} to the ground state ($\geq 10^7\text{ s}^{-1}$ in liquid benzene at room temperature). Thus no fluorescence monitoring of the $B_{2u} \leftarrow A_{1g}$ band in neat benzene at room temperature seems possible. TPOAS is ideal for measuring such weak two-photon spectra in nonfluorescing liquids. The $B_{2u} \leftarrow A_{1g}$ two-photon spectrum we obtain in the liquid resembles the corresponding vapour phase spectrum^{8–13}; similarities include strong intensities of the $14_0^1 1_0^0$ vibronic transitions, laser polarisation dependence, and nearly identical values for ν_{18} , ν_{14} and ν_1 of the $^1B_{2u}$ state in the liquid as in the vapour case. However, the following differences are noted: (1) the electronic origin is $37,724\text{ cm}^{-1}$ in the liquid phase, compared with $38,086\text{ cm}^{-1}$ in the vapour phase; (2) the absorption lines are broader (by a factor of ~ 10) in the liquid as compared with the vapour case^{9,10} so that the rotational branches are unresolved; and (3) since fluorescence quantum efficiencies rapidly drop with higher vibrational excitation⁹ even in the vapour phase, the two-photon spectra by fluorescence monitoring^{8–13} shrink and vanish rapidly at higher vibrational excitation; this does not occur in our case.

The experimental apparatus used includes a scanable Chromatix CMX-4 flash-lamp pumped dye laser which provided the following tuning ranges with the Exciton dyes: Coumarin 2: 448–476 nm; LD 473: 469–500 nm; LD 490: 485–516 nm; Coumarin 504: 502–538 nm. Typical pulse energies of 2 mJ and bandwidth of 2 cm^{-1} with pulse durations of 1 μs were available at 10 pulses s^{-1} . Output beam diameter was 3 mm. A 5-cm focal length lens was used to focus the laser beam at the centre of a cylindrical stainless steel OA cell¹ (1 cm i.d. and 2 cm length), fitted with quartz windows and filled with distilled spectrograde benzene. A quarter-wave plate allows us to convert the normally linearly polarised laser beam to a circularly polarised one for appropriate studies. The transient ultrasonic signal produced by the two-photon absorption was detected by a submersed PZT transducer¹. The transducer

consisted of a 4 mm diameter $\times$ 4 mm high lead zirconate titanate (PZT) cylinder (LTZ-2 from Transducer Products, Connecticut). To obtain good reproducibility, it is essential that there should be no small microbubbles on the transducer face. The transducer signal and the square of the input laser pulse energy were measured with boxcar integrators. part of the dye laser output was analysed with a 2-m grating spectrometer. Either a Rb lamp or Ar laser lines provided markers for accurate calibration.

Figure 1a shows an OA spectrum of benzene in the 18,600 cm^{-1} to 22,200 cm^{-1} region obtained with linearly polarised radiation from the dye laser. The OA signal is normalised to the square of the input power. The seven peaks marked a, b, c, d, e, f, and g are identified as arising from the $B_{2u} \leftarrow A_{1g}$ two-photon absorption. The spacings between the peaks b, c, d, e, f, and g, are approximately 462 cm^{-1} which is in excellent agreement with the expected value of $\nu_1/2$. There are subsidiary small peaks between the major peaks and these are marked by A, B, C, D, and E. Table 1 compares the positions of these peaks with those observed in the high resolution study of two-photon absorption in vapour phase benzene. In order to make a satisfactory comparison, we have to choose the $B_{2u} \leftarrow A_{1g}$ electronic origin at 37,724 cm^{-1} . The identifications and the assignments seem to be consistent and reasonable.

The region of 18,600–22,200 cm^{-1} has two expected weak linear absorption regions arising from the seventh and the eighth harmonic of C—H stretch of benzene. We have studied the seventh harmonic at 18,810 cm^{-1} earlier³. This peak was mistaken as a two-photon peak by Twarowski and Kliger⁵. However, the input power dependence of this peak is found to be 1.05 ± 0.08 which indicates that input power dependence of the various peaks are necessary for correct identifications. Table 1 lists the measured input power dependence of the various peaks, obtained with the 5-cm focal length lens.

To further distinguish between the one-photon and two-photon absorption in benzene in our tuning range, we have also investigated the OA spectrum with no focusing lens. The resultant decrease in the light intensity (by a factor of 10^5) makes two-photon absorption unobservable while the linear absorption signal which is independent of intensity is unaffected. In

these conditions only two absorption peaks are detected by the transducer with amplitudes depending linearly on input power and whose positions are $18,810 \pm 3 \text{ cm}^{-1}$ and $21,040 \pm 7 \text{ cm}^{-1}$. These are identified as the seventh harmonic³ and the eighth harmonic²⁰ of the C—H stretch of benzene, respectively. The peak height of the eighth harmonic at 21,040 cm^{-1} is $\leq 10\%$ of the two-photon peak at 21,031 cm^{-1} observed with the focusing lens. Thus the two-photon spectrum is essentially undistorted by the presence of the underlying single photon absorption.

We now estimate the absolute peak magnitude of the two-photon absorption cross-section for linear polarisation (peak c at 20,106 in Fig. 1a) by comparing it with the one-photon absorption peak X at 18,810 cm^{-1} . At a fixed laser pulse energy of 1.8 mJ, we observe that the ratio of the transducer signals U_c and U_x at peaks c and X respectively is

$$U_c/U_x \approx 3 \quad (1)$$

Since all the nonradiative lifetimes involved are shorter than 100 ns, while the laser pulse is 1 μs long, we assume that all the absorbed light energy is transformed into heat at the end of the laser pulse, causing local thermal expansion, which produces the transducer signal. The amplitude of the transducer signal depends linearly¹ on the light energy absorbed per unit length multiplied by an effective path length l . For the two-photon absorption case, l is taken to be twice the confocal length, that is $l_c \approx 0.05 \text{ cm}$ in our case. For the linear absorption case, l is taken as the laser path length directly in front of the transducer, that is $l_x \approx 0.4 \text{ cm}$. Hence we have

$$U_c \approx K \times \sigma_c N l_c \int \Phi(t) P(t) dt \quad (2)$$

and

$$U_x \approx K \times \alpha_x l_x \int P(t) dt \quad (3)$$

where integrals are performed over the duration of the laser pulse, K is a constant, $N = 6 \times 10^{21} \text{ cm}^{-3}$ is the molecular density, σ_c is the two-photon cross-section at peak c, $\Phi(t)$ is the instantaneous photon flux, $P(t)$ is the instantaneous power, and α_x is the peak absorption coefficient of the seventh harmonic of the C—H stretch, observed³ to be $(4.5 \pm 1.0) \times 10^{-4} \text{ cm}^{-1}$. The

Table 1 Observed peaks by TPOAS for the ${}^1B_{2u} \leftarrow {}^1A_{1g}$ band in liquid benzene

Observed peaks		Power dependence	Cross-section*	$2\nu - 37,724$ (cm^{-1})	Identification†	Calculated‡ position (cm^{-1})
Frequency ν (cm^{-1})	Fig. 1					
18,810	X	1		1-photon 7th harmonic of C—H§		
19,323	a	2	2.1	922	18_0^1	923
19,644	b	2	6.6	1,564	14_0^1	1,564
19,792	A	2	1.5	1,860	$18_0^1 1_0^1$	1,843
20,106	c	2	10	2,488	$14_0^1 1_0^1$	2,488
20,241	B	2	1.8	2,758	$14_0^1 10_0^2$	2,731
20,569	d	2	9.2	3,414	$18_0^1 1_0^2$	2,770
20,690	C	2	2.1	3,656	$14_0^1 1_0^2$	3,414
21,031	e	2	9.0	4,338	$14_0^1 10_0^2 1_0^1$	3,656
21,040	Y	1		4,338	$14_0^1 1_0^3$	4,333
21,139	D	2	3.4	4,554	1-photon 8th harmonic of C—H	
21,487	f	2	4.8	5,250	$14_0^1 10_0^2 1_0^2$	4,562
21,627	E	2	1.7	5,530	$14_0^1 1_0^4$	5,256
21,957	g	2	3.1	6,190	$14_0^1 10_0^2 1_0^3$	5,485
					$14_0^1 1_0^5$	6,179

All values given are for linearly polarised laser light. Listed columns show laser frequency and notation of an absorption peak, the exponent of its laser energy dependence (± 0.1), two-photon absorption cross-section, vibrational energy in the B_{2u} state (the electronic origin was set at 37,724 cm^{-1} in the liquid phase), identification of the transition, and calculated position (above the electronic origin) of the absorption peak assuming gas phase vibrational constants.

* $10^{-52} \text{ cm}^4 \text{ molecule}^{-1} \text{ photon}^{-1} \text{ s}$. The relative values of these cross-sections are accurate to $\pm 10\%$ for the bigger peaks. The uncertainty in the absolute scale is a factor of 3 (see text).

† Using Wilson's notation²¹.

‡ Using Wunsch *et al.*'s constants⁹.

§ Ref. 3.

|| This peak can be seen only without a focusing lens; details of this linear absorption peak is given in ref. 20.

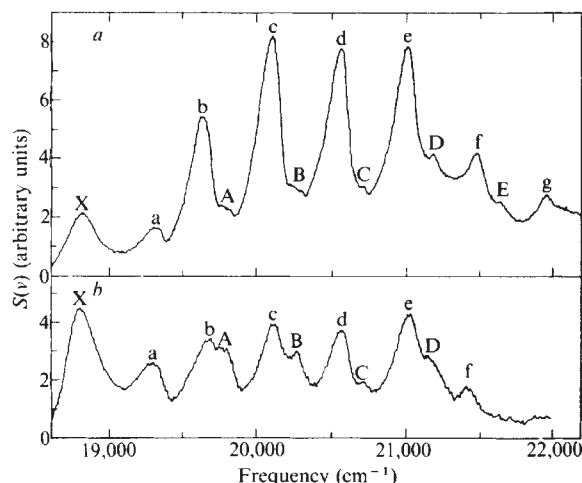


Fig. 1 Normalised two-photon spectra of liquid benzene for the ${}^1B_{2u} \leftarrow {}^1A_{1g}$ band. ν is the single photon energy, and $S(\nu)$ is the transducer signal amplitude normalised to the square of the laser pulse energy. Note that the absorption peak at $18,810 \text{ cm}^{-1}$ is not a member of this band, but is the peak of the seventh harmonic of the C—H stretch. *a*, Spectrum for linear laser polarisation. *b*, Spectrum for circular laser polarisation, magnified by a factor of 2.5 compared with the spectrum in *a*.

laser pulse energy $E = \int P(t) dt$ is fixed at 1.8 mJ and the average $\Phi(t)$ is estimated to be $1.4 \times 10^{27} \text{ photons cm}^{-2} \text{ s}^{-1}$. Note that U_c as given in equation (2) is independent of the focal length of the lens used, since tighter focusing results in a correspondingly shorter confocal length, l_c . This was verified experimentally for focal lengths of 2.5, 5 and 7.5 cm. Combining equations (1)–(3), we estimate that

$$\sigma_c \approx 1 \times 10^{-51} \text{ cm}^4 \text{ molecule}^{-1} \text{ photon}^{-1} \text{ s} \quad (4)$$

with an uncertainty of a factor of 3. The two-photon absorption cross-sections for other peaks in Fig. 1*a* can be scaled appropriately, as given in Table 1.

Judging from the signal-to-noise ratio in Fig. 1*a*, we note that the minimum two-photon cross-section presently detectable is $\sim 10^{-53} \text{ cm}^4 \text{ molecule}^{-1} \text{ photon}^{-1} \text{ s}$. Also, since our OA signal becomes small near $18,500 \text{ cm}^{-1}$ (Fig. 1*a*), we believe that possible broadband spurious effects (such as the laser electrostriction²² effect) are negligible in our present study.

Finally, the use of circular polarisation (as opposed to linear polarisation used in the data reported above) has allowed us to obtain further check on our identification of the two photon transitions listed in Table 1. From vapour phase benzene two-photon absorption data we see that R , defined as the ratio of two photon absorption coefficient for linear polarisation to that for circular polarisation, has well defined values for various vibronic two-photon transitions in benzene. Theoretical considerations show^{9,10,23} that the A_{1g} vibronic transitions should exhibit a ratio R of 5.7 (averaged over the rotational band) while the E_{2g} vibronic transitions should exhibit a ratio R of 0.66; the theory is well supported by experimental investigations in the gas phase^{9,10} and in the crystalline phase²⁴. For the liquid phase, the circularly-polarised TPOAS spectrum is shown in Fig. 1*b*, where the vertical scale is magnified by a factor of 2.5 compared with Fig. 1*a* to bring out the features. Note that (1) the relative heights of the various peaks are quite different and that for the A_{1g} vibronic transitions (for example, peak *b* or *c*) $R \approx 4 \pm 1$, while for the E_{2g} vibronic transitions (for example, peak *a* or *A*) $R \approx 1 \pm 0.3$ in reasonable agreement with theory^{9,22}; and (2) the position of an A_{1g} transition peak is blueshifted (typically by 20 cm^{-1}) for circular polarisation as compared with the linear polarisation case, since different rotational branches are favoured for the different polarisations^{9,10}; however, no shifts are observed for the E_{2g} transition peaks. However, we should

emphasise that most theories of liquids do not treat molecules as being freely rotating and isolated as they would be in the gas phase, and hence the comparison between the effect of rotational structure in gas phase and liquid phase may not be strictly valid.

We have shown that TPOAS, utilising pulsed lasers and submersed transducers, represents a very powerful tool for the study of two-photon absorption spectra of non-fluorescing liquids. By this method, accurate two-photon spectroscopy of the ${}^1B_{2u}$ state of benzene in the neat liquid is studied with quantitative cross-sections derived for the first time. The selection rules for two-photon absorption are very different from those for one-photon absorption (at about twice the photon energy). Thus the TPOAS allows us to find and to determine the positions of vibronic levels not possible in single photon spectroscopy. We plan to extend these studies to multiphoton absorption and ionisation phenomena in various liquids.

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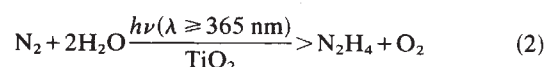
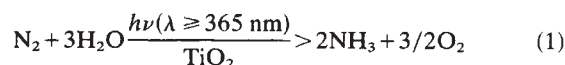
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1. Patel, C. K. N. & Tam, A. C. *Appl. Phys. Lett.* **34**, 467 (1979).
2. Tam, A. C., Patel, C. K. N. & Kerl, R. J. *Opt. Lett.* **4**, 81 (1979).
3. Patel, C. K. N. & Tam, A. C. *Chem. Phys. Lett.* (in the press).
4. Patel, C. K. N. & Tam, A. C. *Appl. Phys. Lett.* (in the press).
5. Twarowski, A. J. & Klinger, D. S. *Chem. Phys.* **20**, 253 (1977); **20**, 259 (1977).
6. Bonch-Bruевич, A. M., Razumova, T. K. & Starobogatov, I. O. *Opt. Spectrosc.* **42**, 45 (1977).
7. Herzberg, G. *Molecular Spectra and Molecular Structure*, Vol. 3 (Van Nostrand, New York, 1966).
8. Hochstrasser, R. M., Wessel, J. E. & Sung, N. H. *J. chem. Phys.* **60**, 317 (1974).
9. Wunsch, L., Neusser, N. J. & Schlag, E. W. *Chem. Phys. Lett.* **31**, 433 (1975); **32**, 210 (1975).
10. Wunsch, L., Metz, F., Neusser, H. J. & Schlag, E. W. *J. chem. Phys.* **66**, 386 (1977).
11. Friedrich, D. M. & McClain, W. M. *Chem. Phys. Lett.* **32**, 541 (1975).
12. Lombardi, J. R., Friedrich, D. M. & McClain, W. M. *Chem. Phys. Lett.* **38**, 213 (1976).
13. Bray, R. G., Hochstrasser, R. M. & Sung, H. N. *Chem. Phys. Lett.* **33**, 1 (1975).
14. Lombardi, J. R., Wallenstein, R., Hansch, T. W. & Friedrich, D. M. *J. chem. Phys.* **65**, 2357 (1976).
15. Berlman, I. B. *Handbook of Fluorescence Spectra of Aromatic Molecules* (Academic, New York, 1965).
16. Parmenter, C. S. & Rau, J. D. *J. chem. Phys.* **51**, 2242 (1969).
17. Thomas, J. K. A. *Rev. Phys. Chem.* **21**, 17 (1970).
18. Bensasson, R. V., Richards, J. T. & Thomas, J. K. *Chem. Phys. Lett.* **9**, 13 (1971).
19. Hentz, R. R. & Thibault, R. M. *J. phys. Chem.* **77**, 1105 (1973).
20. Patel, C. K. N., Tam, A. C. & Kerl, R. J. *J. chem. Phys.* (in the press).
21. Wilson, E. B., Jr. *Phys. Rev.* **45**, 706 (1934).
22. Bechuk, A. S., Mizin, V. M. & Salova, N. Ya. *Opt. Spectrosc.* **44**, 91 (1978).
23. Bray, R. G. & Hochstrasser, R. M. *Molec. Phys.* **31**, 412 (1976).
24. Hochstrasser, R. M., Klimcak, C. M. & Meredith, G. R. *J. chem. Phys.* **70**, 870 (1979).

Photocatalytically induced fixation of molecular nitrogen by near UV radiation

A MAJOR method whereby molecular nitrogen is fixed in the soil is through microorganisms¹. Recently, however, molecular nitrogen has been shown² to react with water vapour in the presence of TiO_2 under the stimulus of near UV radiation to produce ammonia and hydrazine according to the photoreducing reactions summarised as:



We report here the results of some recent experiments in which molecular nitrogen has been fixed, at room temperature in oxidising conditions, on the surface of rutile (TiO_2) by low-energy UV photons.

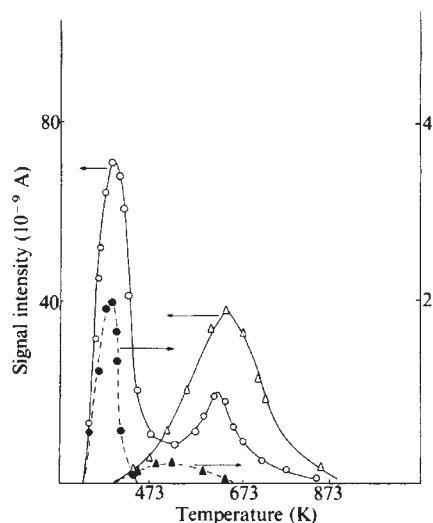
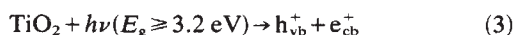


Fig. 1 Temperature programmed desorption profiles of TiO_2 surfaces: solid line, following the interaction of 20 Nm^{-2} of molecular nitrogen with a surface saturated with pre-adsorbed hydrogen peroxide; dashed line, following exposure to UV radiation ($\lambda \geq 365 \text{ nm}$) in the presence of air. (The saturated surface containing $\sim 1.2 \times 10^8 \text{ molecule m}^{-2}$ of H_2O_2 was produced by adding an aqueous solution of H_2O_2 (Analar 30% w/v) to the outgassed rutile surface, followed by evacuation to remove the excess liquid.) $\circ$, $m/e = 32$; $\triangle$, $m/e = 30$. For solid symbols, read right-hand scale; for open symbols, read left-hand scale.

TiO_2 powders, which contain adsorbed water, show significantly greater photochemical reactivity towards oxygen than specimens which have been dehydroxylated. It has been suggested³ that the adsorbed hydroxyl groups, OH_{ads}^+ , act as traps for the positive holes, h_{vb}^+ , which are formed in the valence band of the TiO_2 when it is irradiated with photons of energy equal to or greater than the magnitude of the electronic band gap ($E_g(\text{TiO}_2) \sim 3.2 \text{ eV}$) according to the mechanism:



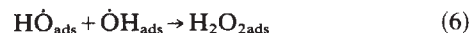
The hole trapping leaves the conduction band electron, e_{cb}^- , available to interact with the ambient atmosphere and the chemisorption of molecular oxygen takes place according to:



Much information has been obtained about the nature of the adsorbed species $\text{O}_{2\text{ads}}^-$ (ref. 4), but the identification of the hydroxyl radical has been much more difficult to confirm due to its high reactivity, although there is indirect evidence⁵ of its existence.

In a recent series of experiments we have used the technique of temperature programmed desorption (TPD) in conjunction with mass spectrometry and gas-liquid chromatography to investigate the surfaces of rutile powders, which contain pre-adsorbed water, following their exposure to prolonged irradiation (300 min) with near UV photons ($\lambda \geq 365 \text{ nm}$) at room temperature in the presence of either pure oxygen or air. (These rutile powders, supplied by Tioxide International Ltd, contained 99% rutile with a specific surface area of $13 \text{ m}^2 \text{ g}^{-1}$ as determined by krypton adsorption at 78 K.)

A comparison of the desorption spectra from these surfaces, following the irradiation treatment, with that arising from a TiO_2 surface containing pre-adsorbed hydrogen peroxide only, shows a correspondence between the molecular oxygen peaks in the respective profiles (Fig. 1). These results indicate that hydrogen peroxide is formed on the surface of the TiO_2 powder during the irradiation, probably through the direct interaction of the hydroxyl radicals which form during the hole trapping process:



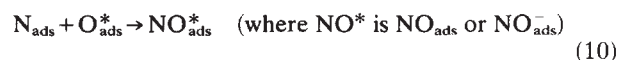
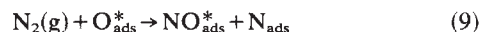
A remarkable aspect of the desorption spectra from these surfaces is the appearance of a gaseous molecular species which from its mass cracking pattern ($m/e = 30, 14, 16$) and the relative peak intensity ratios of 100:6:1 has been identified as nitric oxide. In the case of surfaces containing pre-adsorbed hydrogen peroxide the nitric oxide appears only after exposing the surface to molecular nitrogen or to air. Similar effects are observed if the TiO_2 surfaces are irradiated in pure oxygen, whereas it seems that the nitric oxide forms directly if the irradiation takes place in air or a nitrogen-oxygen mixture. The quantity of nitric oxide desorbed from the surface saturated with pre-adsorbed hydrogen peroxide (Fig. 1) corresponds to a coverage of approximately $3 \times 10^{17} \text{ molecule m}^{-2}$.

The surface species from which the nitric oxide arises has not yet been identified but it has been clearly demonstrated that molecular nitrogen reacts with adsorbed hydrogen peroxide (Fig. 2) and it is therefore most probable that nitric oxide arises from the direct interaction of molecular nitrogen with a species originating from the adsorbed hydrogen peroxide. Moreover it had been established that hydrogen peroxide is formed on the surface of TiO_2 (rutile) when oxygen, water and UV radiation are present simultaneously and that molecular nitrogen can be fixed at the surface of the TiO_2 through its interaction with the hydrogen peroxide which has been photosynthesised *in situ*.

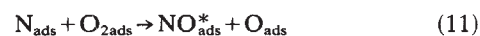
Molecular nitrogen is thought to react with a species of atomic oxygen, either $\text{O}_{\text{ads}}^\circ$ or O_{ads}^- , which originates from either the thermal or photochemical decomposition of the adsorbed hydrogen peroxide, as the interaction of molecular nitrogen with hydroxyl radicals is energetically very unfavourable⁶,



We believe that the adsorbed species from which the gaseous nitric oxide originates is probably an adsorbed form of molecular nitric oxide, or one of its ions, and is created by a mechanism of the type shown in equations (8)–(11):



or



X-ray photoelectron spectroscopic (XPS) examinations of the surfaces of these TiO_2 specimens show nitrogen (1s) signals at a

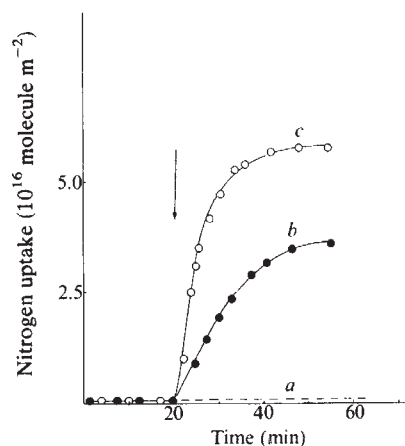


Fig. 2 The interaction of molecular nitrogen with hydrogen peroxide vapour at the surface of TiO_2 (rutile) at 298 K. *a*, TiO_2 absent; *b*, initial pressure of nitrogen = 3.3 N m^{-2} ; *c*, initial pressure of nitrogen = 20.0 N m^{-2} . Arrow indicates hydrogen peroxide vapour admitted.

binding energy of 399.5 eV, confirming the presence of an adsorbed species containing nitrogen. In the experiment with pre-adsorbed hydrogen peroxide, the saturation coverage of the nitrogen species was estimated from the XPS data to be of the order of 10% of a monolayer, but it is not possible to decide whether the N(1s) signal originates from adsorbed nitric oxide or from a diatomic nitrogen species. Nitric oxide adsorption at 298 K (ref. 7), on oxidised TiO₂ surfaces, produces two irreversible states of adsorption having maxima in the desorption profile at 450 K and 660 K respectively. It is the stronger state of adsorption of nitric oxide to which the fixed nitrogen most closely corresponds. Electron paramagnetic resonance studies of the samples containing the fixed nitrogen failed to detect any species which could be ascribed to adsorbed molecular nitric oxide and we conclude that the adsorbed species is probably NO_{ads}⁻ (or N₂O_{2ads}²⁻) as enhancement in the uptake of molecular nitrogen suppresses the formation of the species, O_{2ads} (ref. 7) which arises from the decomposition of the adsorbed hydrogen peroxide. In addition no mass spectrometric evidence has been found in the temperature programmed desorption profiles for the existence of molecules of N₂O or NO₂.

Titanium dioxide surfaces are probably not unique in being able to participate in the photocatalytic fixation of molecular nitrogen as many other solids exhibit photocatalytic properties⁸. The adsorbed nitrogen species formed during the fixation reaction may itself undergo further oxidation to a more stable species, such as the NO₂⁻ ion⁹, and it seems likely that these processes can occur naturally at the Earth's surface under the stimulus of sunlight.

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1. Jones, K. in *Comprehensive Inorganic Chemistry*, Vol. 2, Ch. 19 (ed. Trotman-Dickenson, A. F.) (Pergamon, Oxford, 1973).
2. Schrauzer, G. N. & Guth, T. D. *J. Am. chem. Soc.* **99**, 7189 (1977).
3. Bickley, R. I. & Stone, F. S. *J. Catal.* **31**, 389 (1973).
4. Meriaudeau, P. & Vedrine, J. C. *JCS Faraday I*, **72**, 472 (1976).
5. Voltz, H. G., Kämpf, G. & Fitzky, M. G., *Farbe & Lack* **78**, 1037 (1972).
6. Benson, S. W. *Thermochemical Kinetics* (Wiley, New York, 1968).
7. Bickley, R. I. & Vishwanathan, V. *JCS Faraday I* (to be submitted).
8. Bickley, R. I. *Chemical Physics of Solids and their Surfaces*, Vol. 7 (Chemical Society, London, 1978).
9. Maclean, W. & Ritchie, M. *J. appl. Chem.* **15**, 452 (1965).

CS₂ and COS in the stratospheric sulphur budget

THE persistence and pervasion of the stratospheric sulphate (Junge) layer^{1,2} during periods of low volcanic activities have puzzled many aeronomers. Junge³ suggested that sulphur dioxide, SO₂, is probably the gaseous precursor for the sulphate layer. The origin of stratospheric SO₂ is, however, uncertain. The gas may originate from the troposphere through upward diffusion processes³, while Crutzen⁴ recently suggested that the presence of SO₂ in the stratosphere may be attributed to photolysis of carbonyl sulphide, COS. Recent measurements⁵ showed that the mixing ratio of SO₂ remains remarkably constant in the upper troposphere and the lower stratosphere region. We show here that such peculiar phenomena may be explained by oxidation of COS and carbon disulphide, CS₂. The gas, CS₂ has been detected by Lovelock⁶ in seawater samples, and by Sandalls and Penkett⁷ in the lower troposphere. We⁸

Table 1 Chemical model

Ref	Reaction	Rate constant*
9, 10	CS ₂ + OH → COS + HS	R1 = 1.9(-13)§
11	CS ₂ + O → CS + SO → COS + S → CO + S ₂	R2 = 3.7(-11) exp [-700/T] (fa = 0.8, fb = 0.1, fc = 0.1)†
12	CS ₂ + hν → CS + S	J3‡
26	COS + hν → CO + S	J4‡
9, 10	COS + OH → CO ₂ + HS	R5 = 5.7(-14)§
27	COS + O → CO + SO	R6 = 1.6(-11) exp [-2250/T] 8.2(-13) × [M]
17	SO ₂ + OH + M → HSO ₃ + M	R7 = 7.9(+17) + [M]
28	SO ₂ → precipitation and scavenging	R8 = 5.8(-7)Z < 10 km

* The rate constants are in units of cm³ s⁻¹ for two-body reaction, cm⁶ s⁻¹ for three-body reaction and s⁻¹ for photolysis rates and R8.

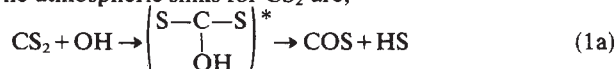
† fa, fb and fc are the relative yields for the three branches.

‡ The photolysis rates are calculated from measured cross-sections allowing properly for diurnal variation of solar intensity.

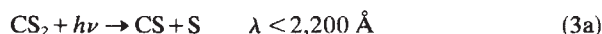
§ These are room temperature values⁹. The activation energy for R1 and R5 should be negligible based on proposed reaction mechanism¹⁰.

recently argued that CS₂ may be a precursor for atmospheric COS. Here we investigate the possible role of COS and CS₂ in the stratospheric sulphur budget and account for the source for stratospheric sulphate, the observed distributions of SO₂ and COS.

The atmospheric sinks for CS₂ are,



and



Reaction (1a) is exothermic while reaction (1b) is endothermic⁹. Recent work by Kurylo and Laufer¹⁰ indirectly supports reaction (1a). Reaction (2) dominates in the stratosphere and proceeds¹¹ primarily through reaction (2a). Photolysis of CS₂ (reaction (3a)) seems to be relatively unimportant according to

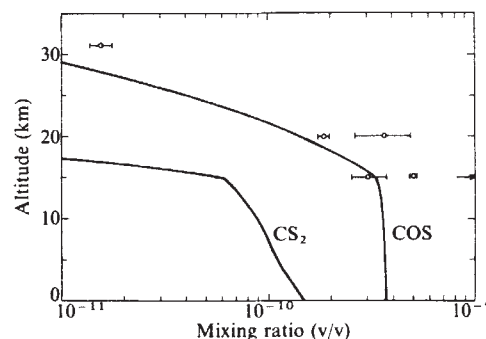


Fig. 1 Computed vertical profiles of CS₂ and COS. The profiles are calculated using a one-dimensional model described previously²⁰. The rate constants for O—C—N—Cl—S chemistry are from ref. 29 and the main sulphur reactions are given in Table 1. The diffusion coefficients are from ref. 30. The boundary conditions at 0 km are 0.15 p.p.b. for CS₂ and zero flux for COS. Observed CS₂ and COS surface concentrations range from 0.07 to 0.37 p.p.b. (ref. 7) and 0.3 to 0.5 p.p.b. (refs 7, 21, 22) respectively. The stratospheric COS data³¹ (○) are plotted for comparison. The arrow indicates the position of the tropopause.

absorption cross-section data¹². Photo-excitation of CS₂ (reaction (3b)) and the subsequent chemistry involving CS₂^{*} could lead to additional production of COS and SO₂ (refs 13, 14). Reactions involving CS₂^{*} have not been included in this study due to lack of laboratory data. Reaction (1a) could provide a major source for COS. We assume that reaction products such as HS, CS and S would ultimately be oxidised to form SO₂ over a relatively short time, although reaction of CS with O₂ could provide an additional source for COS (ref. 15). Chemical removal of SO₂ in the stratosphere is dominated¹⁶⁻¹⁸ by the reaction,



The main chemical processes are summarised in Table 1. The rate constants *R*1 and *R*5 (Table 1) used are from Kurylo⁹. These values⁹ apparently conflict with the corresponding upper limits set by Atkinson *et al.*¹⁹, whose measurements were performed in less stringent conditions (higher reactant pressure). The observed¹⁹ nonlinear dependence of first-order OH decay rates on reactant concentration may be attributed to complications⁹ associated with secondary reactions.

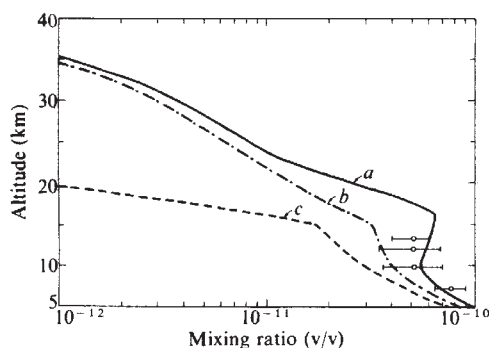


Fig. 2 Computed vertical profiles of SO₂, (lines) and measurements (circles)⁵. Curve *a* is calculated using the full model described in Table 1. Curve *b* is calculated with CS₂ chemistry omitted. Curve *c* is calculated with both COS and CS₂ chemistry omitted. In all three curves, a value of 0.3 p.p.b. is assumed for SO₂ at 1 km. This boundary is chosen such that the integrated tropospheric abundance of SO₂ is equal to 6×10^5 (S) ton, a value consistent with the global estimates of 4.5×10^5 and 5.2×10^5 (S) ton given by Mészáros³² and Friend³³, respectively. The fixed mixing ratio boundary condition at 1 km implicitly incorporates²⁸ the effects of any production processes (for example, H₂S oxidation, anthropogenic and volcanic emissions) and removal processes (for example dry deposition) occurring below 1 km. Note that the reported data⁵ were taken at ~50° N, while our model corresponds to 30° N. For proper comparison, the data points should be shifted upwards by ~2 km (ref. 30).

The vertical distribution of a minor species may be obtained by solving the appropriate one-dimensional continuity and flux equations²⁰. Figure 1 shows the calculated altitude profiles of CS₂ and COS. The calculated mixing ratio of CS₂ drops abruptly above ~14 km altitude, reflecting the onset of the relatively fast reaction of CS₂ and O atoms. In calculating the COS profile, we specify a zero flux surface boundary condition instead of using its mixing ratio at the boundary, an assumption consistent with our premise that CS₂ is a precursor⁸ of COS. The apparent agreement between the predicted and observed surface COS mixing ratios^{7,21,22} of about 0.4 p.p.b. may be fortuitous in view of the possible ambiguities in rate constant data (*R*1 and *R*5 in Table 1) and paucity of CS₂ data. Nevertheless, an approximate balance in COS budget⁸ may be achieved so long as the relationship

$$\frac{[\text{CS}_2]}{[\text{COS}]} \approx \frac{R5}{R1} \quad (5)$$

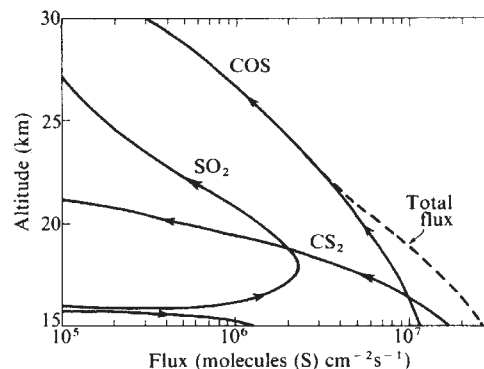


Fig. 3 Computed fluxes for CS₂, COS and SO₂. The flux of CS₂ has been multiplied by 2, reflecting that each molecule carries two sulphur atoms. The total flux Φ_t is set equal to $2\phi_{\text{CS}_2} + \phi_{\text{COS}} + \phi_{\text{SO}_2}$. The arrows indicate direction of flow.

holds in the lower troposphere. Otherwise, alternative sources and/or sinks for COS must be found to account for the observed surface concentrations of COS. If *R*1 were much less than the value⁹ used here, CS₂ would remain relatively well mixed in the troposphere, making it a more potent source of stratospheric SO₂.

Figure 2 shows the altitude profiles of SO₂ and data reported by Jaeschke⁵. These three curves correspond to different assumptions concerning the atmospheric sources of SO₂. Curve *c* illustrates the case where there is no *in situ* source for SO₂. The rapid decrease in mixing ratio reflects the rapid removal of SO₂ by reaction (4). Curve *a* (standard model) represents the full model calculation where SO₂ is produced locally by reactions *R*1 through *R*6 in Table 1. Our calculation showed that SO₂ (curve *a*) is relatively well mixed in the region 10–15 km, agreeing with observed data⁵, although such a feature could be accounted for by upwelling air motion during particular weather conditions of the day⁵. Note that in the absence of a CS₂ source, the calculated SO₂ distribution (curve *b*) lies somewhat below the observed data⁵.

Figure 3 shows the combined flux, Φ_t , of CS₂, COS and SO₂ of about 2.5×10^7 molecules (S) cm⁻² s⁻¹ or 2.1×10^5 ton (S) yr⁻¹ into the stratosphere. This value should be compared with several independent estimates^{3,23,24} of 5×10^6 to 2×10^7 molecules (S) cm⁻² s⁻¹ necessary to sustain the sulphate layer. Note that the major contribution to Φ_t comes from CS₂ and COS, with each gas dominating at different altitude regions. The rapid increase of SO₂ flux with altitude between 15 and 17 km reflects the *in situ* production of SO₂ in the model rather than upward diffusion³ of SO₂ from the troposphere. It seems that the persistence of the sulphate layer may be attributed partly to the oxidation of CS₂ below ~20 km and partly to the oxidation of COS above ~20 km, and that the observed altitude^{3,23} (~20 km) at which the sulphate layer peaks may be modulated by the relative strengths as well as the altitude dependence of these two sources.

The calculated global flux of CS₂ is about 6.4×10^8 molecules (S) cm⁻² s⁻¹ or 5.4×10^6 ton (S) yr⁻¹, implying a CS₂ life time of about 0.2 yr compared with that of COS of about 0.6 yr. The calculated flux depends on the tropospheric OH concentration, CS₂ boundary condition and rate constant *R*1, all of which are subject to large uncertainties. The measured concentration of CS₂ in seawater samples reported by Lovelock⁶ suggests that the ocean might provide a source of CS₂ of order 10^7 molecules (S) cm⁻² s⁻¹ based on a simple piston model²⁵. The role of the ocean in the atmospheric CS₂ budget has yet to be determined.

In conclusion, CS₂ could have an important role in the stratospheric sulphur budget. Clearly, more atmospheric measurements of SO₂, CS₂ and COS, as well as accurate determination of rate constants for *R*1 and *R*5, are needed for model validation. It is also evident that other sources of CS₂ and COS need to be identified.

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1. Junge, C. E., Chagnon, C. W. & Manson, J. E. *J. Met.* **18**, 81 (1961).
2. Castleman, A. W. Jr, Munkelwitz, H. R. & Manowitz, B. *Tellus* **26**, 222 (1974).
3. Junge, C. E. *Proc. IAMAP Conf. on Structure, Composition and General Circulation of Upper and Lower Atmos.* 85-97 (1974).
4. Crutzen, P. J. *Geophys. Res. Lett.* **3**, 73 (1976).
5. Jaeschke, W., Schmitt, R. & Georgii, H.-W. *Geophys. Res. Lett.* **3**, 517 (1976).
6. Lovelock, J. E. *Nature* **248**, 625 (1974).
7. Sandalls, F. J. & Penkett, S. A. *Atmos. Environ.* **11**, 197 (1977).
8. Sze, N. D. & Ko, M. K. W. *Nature* **278**, 731 (1979).
9. Kurylo, M. J. *Chem. Phys. Lett.* **58**, 238 (1978).
10. Kurylo, M. J. & Laufer, A. H. *J. Chem. Phys.* **70**, 2032 (1979).
11. Baulch, D. L., Drysdale, D. D. & Horne, D. G. *Evaluated Kinetic Data for High Temperature Reactions Vol. III* (Butterworths, London, 1976).
12. Rabalais, J. W., McDonald, J. M., Scherr, V. & McGlynn, S. P. *Chem. Rev.* **71**, 73 (1971).
13. DeSorgo, M., Yarwood, A. J., Strausz, O. P. & Gunning, H. E. *Can. J. Chem.* **43**, 1886 (1965).
14. Wood, W. P. & Heicklen, J. J. *J. phys. Chem.* **75**, 854 (1971).
15. Breckenridge, W. H., Kolln, W. S. & Moore, D. S. *Chem. Phys. Lett.* **32**, 290 (1975).
16. Davis, D. D. *Can. J. Chem.* **52**, 1405 (1974).
17. Moortgat, C. K. & Junge, C. E. *Pageoph* **115**, 759 (1977).
18. Cox, R. A. *Phil. Trans. R. Soc. A290*, 543 (1979).
19. Atkinson, R., Perry, R. A. & Pitts, J. N. Jr *J. Chem. Phys. Lett.* **54**, 14 (1978).
20. Sze, N. D. & Wu, M. F. *Atmos. Environ.* **10**, 1117 (1976).
21. Hanst, P. L., Spiller, L. L., Watts, D. M., Spence, J. W. & Miller, M. F. *J. Air Pollut. Control. Ass.* **25**, 1220 (1975).
22. Maroulis, P. J., Torres, A. L. & Bandy, A. R. *Geophys. Res. Lett.* **4**, 510 (1977).
23. Lazrus, A. L. & Gandrud, B. W. *J. geophys. Res.* **79**, 3424 (1974).
24. Cadle, R. D. *J. geophys. Res.* **80**, 1650 (1975).
25. Broecker, W. S. & Peng, T.-H. *Tellus* **26**, 21 (1974).
26. Breckenridge, W. H. & Taube, H. *J. chem. Phys.* **52**, 1713 (1970).
27. Krezenski, D. C., Simonaitis, R. & Heicklen, J. *Int. J. chem. Kin.* **3**, 467 (1971).
28. Gravenhorst, G., Janssen-Schmidt, T. & Ehhalt, D. H. *Atmos. Environ.* **12**, 691 (1978).
29. Hudson, R. D. (ed.) *Chlorofluoromethane and the Stratosphere* (NASA, Washington, D.C. 1977).
30. Wofsy, S. C. A. *Rev. Earth Planet. Sci.* **4**, 441 (1976).
31. Inn, E. C. Y., Vedder, J. F. & Tyson, B. J. *Geophys. Res. Lett.* **6**, 191 (1979).
32. Mészáros, E. *Atmos. Environ.* **12**, 699 (1978).
33. Friend, J. P. *Chemistry of the Lower Atmosphere* (ed. Rasool, S. J. (Plenum, New York, 1973).

Thermohaline intrusions lie across isopycnals

A MAJOR task of physical oceanography is the determination of the fluxes of heat and salt and the identification of the processes responsible for producing the fluxes. For the development of realistic models of the ocean it is particularly important that fluxes across surfaces of constant density (isopycnal surfaces) be distinguished from those along isopycnals. We report here direct evidence that at least some of the 5–20-m thick intrusions commonly found in regions of strong horizontal variability (fronts) lie across isopycnals. The intrusions are formed by primarily lateral advection to produce complicated interleavings of the differing types of water across the fronts. The ubiquitous occurrence of intrusions in frontal regions and the fact that they cross isopycnal surfaces indicates that they are important factors in the vertical, as well as the horizontal, flux budgets of heat and salt. The low levels of cross-isopycnal fluxes due to small scale turbulence away from fronts¹ further suggests that these intrusions may be important in the overall global flux budgets of heat and salt.

The intrusions were studied at Ocean Station P (50° N, 145° W) in August 1977 during the mixed layer experiment (MILE). One of the reasons for selecting the site was its distance from known fronts. However, repeated surveys with a rapidly profiling towed vehicle revealed fronts in the weakly stratified region between 40 m (the base of the seasonal thermocline) and 100 m (the beginning of a strong salinity increase or halocline). The fronts were characterised by lateral changes along isopycnal-surfaces of 0.4 °C and 0.05‰ over distances < 2 km.

Tongues of water up to 8 km long, with widths of 1 km and thicknesses of 10–20 m, were observed to intrude into the warmer, more saline water from the cool, low salinity side of one of the fronts. Figure 1 shows the down profiles from a tow section across a tongue. The section was roughly parallel to the front so the section shows the tongue in the direction of its minimum horizontal extent. Minima in temperature and salinity appear in the 7th profile between 0.5 and 0.55 MPa (these pressures correspond to nearly 50 m in depth) and slope downwards towards the right. The structure also becomes thicker as it deepens and shows splitting into double minima. The density changes due to the matched temperature and salinity structures are so nearly compensating that little evidence for the feature is apparent in the bottom set of profiles.

To be certain that the slope is not due to a changing internal wave displacement, the temperature and salinity contours must be examined as functions of potential density rather than of pressure. The quality of the density profiles is central to this argument. Careful laboratory tests of the temperature and conductivity sensors^{2,3} show that the r.m.s. random noise in the data is equivalent to $2.1 \times 10^{-4} \text{ kg m}^{-3}$, which is negligible for our purpose. The dynamic response of the temperature probe as it passed through a thin plume was obtained in the laboratory. The response of the conductivity cell, determined by comparison of the temperature-salinity diagrams from up and down depth cycles, showed that the cell response was closely matched to that of temperature. Even so, a numerical simulation of the sensors passing through features similar to those in Fig. 1 revealed that density errors equivalent to displacements of 1–3 m could occur as a consequence of microstructure in the profile. However, this is much less than the 15 m vertical displacement of feature across the tow path.

Contours of isotherms plotted in Fig. 2 with potential density as the vertical coordinate show the temperature minimum sloping with respect to lines of constant density. This figure includes data preceding that in Fig. 1, showing the nearly level isotherms in a region where an intrusion is not present. As a further test of the data, additional contours were made using only the up traces. If the slope were an artefact of a dynamic response

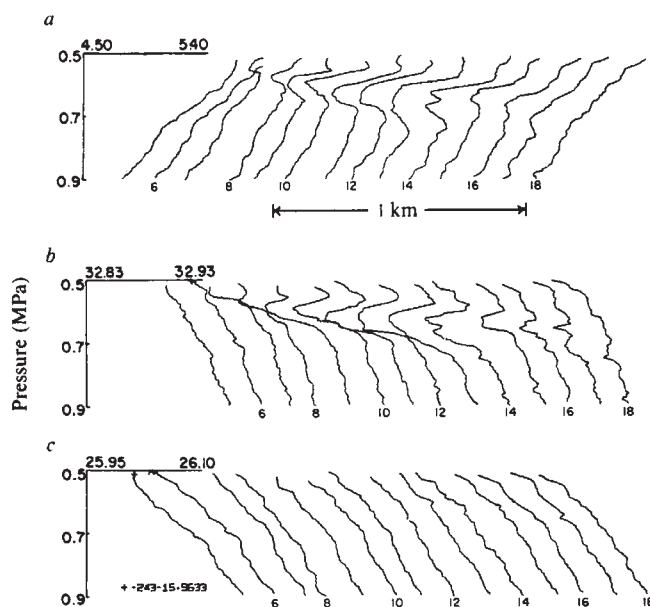


Fig. 1 Successive down profiles of temperature (a), salinity (b) and density (c) from a depth-cycling towed vehicle (1 MPa corresponds to a depth interval of 99.2 m). Pronounced minima in temperature and salinity slope downward from left to right and mark an intrusion of cool, low salinity water into the profile. The density effects of the cooler (an increase in density), low salinity (a decrease in density) water are so nearly compensating that there is little evidence for the feature in the bottom set of profiles.

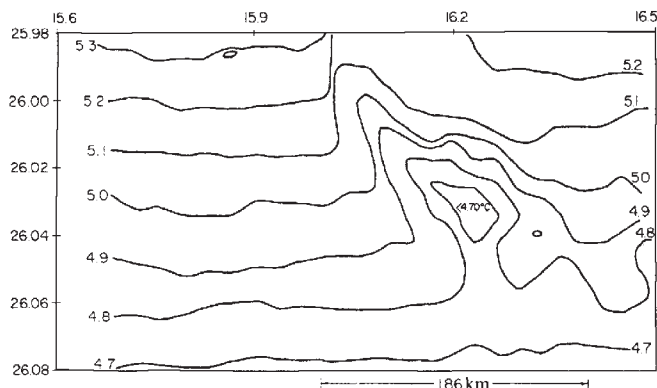


Fig. 2 Isotherms plotted with potential density (in kg m^{-3}) as the vertical coordinate. Internal wave distortions are removed from the contour. The remaining structure is an intrusion of cool, low salinity water that slopes downward from left to right.

problem, the slope should have reversed in the up casts. This is not the case and the maps are nearly identical to Fig. 2.

A section made at a 60° angle to that shown in the figures showed that the intrusion was also sloping downwards across isopycnal surfaces in a direction away from the front. Microstructure profiles through similar intrusions⁵ have revealed distinctive signatures on the upper and lower boundaries of the minima that have been identified as the appropriate type of double-diffusive convection.

Double-diffusive convection is a consequence of the much lower molecular diffusivity of salt dissolved in water compared to the thermal diffusivity⁶. Consequently, when warm, saline water overlies cool, fresh water in a stratified profile small perturbations lead to a series of centimetre thick rising and descending columns known as salt fingers. When warm, saline water occurs beneath cooler, fresher water (known as the diffusive regime), a sequence of convecting regions separated by their interfaces is formed. Although the observations in Fig. 1 did not have the spatial resolution to detect the signatures of double diffusion, the inference from the microstructure observations is that salt fingering occurred above the minima and the diffusive regime below. Laboratory experiments⁷ of diffusively unstable intrusions have shown that the increase in density below a salt fingering region dominates the decrease above the 'diffusive' regime, with the consequence that cool, fresh intrusions tend to sink as they move laterally. The thickening and formation multiple minima are also revealed in the laboratory work.

The cross-isopycnal slope of 100 m-thick intrusions across the Antarctic Front has been inferred⁷ by a cross-spectral comparison of horizontally separated profiles. We have obtained the same result directly for much thinner features by mapping against density.

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1. Gregg, M. C. *J. phys. Oceanogr.* **7**, 436 (1977).
2. Pederson, A. M. *Trans. Mar. Technol. Soc. Mar. Temp. Measurements Symp.* 135 (1969).
3. Pederson, A. M. & Gregg, M. C. *I.E.E.E. J. of Oceanic Engng.* (in the press).
4. Dantzer, H. L. *Deep Sea Res.* **21**, 675 (1974).
5. Gregg, M. C. & Cox, C. S. *Deep Sea Res.* **19**, 355 (1972).
6. Turner, J. S. *Buoyancy Effects in Fluids* (Cambridge University Press, 1973).
7. Turner, J. S. & Chen, C. F. *J. Fluid Mech.* **63**, 577 (1974).
8. Joyce, T. M., Zenk, W. & Toole, J. M. *J. geophys. Res.* **83**, 6093 (1978).

Connectance in real biotic communities and critical values for stability of model ecosystems

BIOLOGICAL communities differ in the number of species that they contain; in the number of interactions between species (primarily feeding and competitive relationships); and in the intensity of their interactions. We present here the first extensive empirical data on the relationship between connectance (defined as the fraction of the pairs of species which directly interact^{1,2}) and species richness in biological communities. Our results show that connectance may decline considerably as the number of species increases.

A decade ago, most ecologists firmly believed that increased ecosystem complexity (both the number of species and the number of interactions) would lead to greater ecosystem stability. For example, Odum³ suggested that "Higher diversity, then, means longer food chains and more cases of symbiosis (mutualism, parasitism, commensalism, and so forth), and greater possibilities for negative feedback control, which reduces oscillations and hence increases stability". This attractive and widespread statement has been shaken dramatically by May^{1,2,4,5} and his followers⁶⁻¹³. May examined the complexity-stability question in model ecosystems by constructing interaction matrices with each off-diagonal element (interaction coefficient) assigned from a distribution of random numbers, where distribution itself has mean value zero, and standard deviation value (interaction strength) s ; the stability of the matrices was then evaluated in terms of their eigenvalues¹⁴. The central feature of May's results for model ecosystems is the sharp transition from stable to unstable behaviour as soon as either the species number m , or the connectance C , or the interaction strength s , exceeds a critical value. If the equation $s\sqrt{mC} > 1$ holds, the system will almost certainly be unstable. In general, by becoming more complex a system becomes less likely to be stable. Selected empirical evidence supports such a conclusion. According to May⁴, "this inverts the naive, if well-intentioned, view that 'complexity begets stability', and its accompanying moral that we should preserve, or even create, complex systems as buffers against man's importunities". May's corollaries are useful for sharpening discussion but their application to natural resource management and optimum land use is not without danger.

Recently, Lawlor¹⁵ has shown that severe constraints must be introduced on the structure of an interaction matrix if it is to represent a biologically acceptable system. He has suggested, *inter alia*, the hyperbolic reduction of the connectance C as the number of species m increases: $Cm = 10$. This relationship ensures stability if the standard deviation s of the interaction coefficients (not variance; this is the mistake of Lawlor), is less than about one-third. Lawlor's comments induce the urgent question: What is the true connectance of real communities?

The question seems to be unanswerable on the basis of very rough, speculative or fragmentary data in most of the literature, even if such data are sometimes used in theoretical considerations. The extensive data collected by one of us^{16,17} have allowed reconstitution of the food webs in a great number of plant-aphid-parasitoid communities. The parasitoids belong to three families: Aphidiidae, Aphelinidae and Encyrtidae (Hymenoptera). All host-parasitoid relationships have been tested in the laboratory. The results for one representative community are demonstrated in Fig. 1. In the canopy of an oak-pine-birch forest (the order Pino-Quercetalia Soó 1962 in the sense of the Zürich-Montpellier phytosociological school) 37 species have been recorded of vascular plants, aphids and their parasitoids; a trophic connectance of 0.059 has been calculated for this community. Some chains have been confirmed by numerous field assessments, for example, 23 geographically distinct observations for that of *Pinus silvestris*-*Schizolachnus pineti*-*Pauesia*

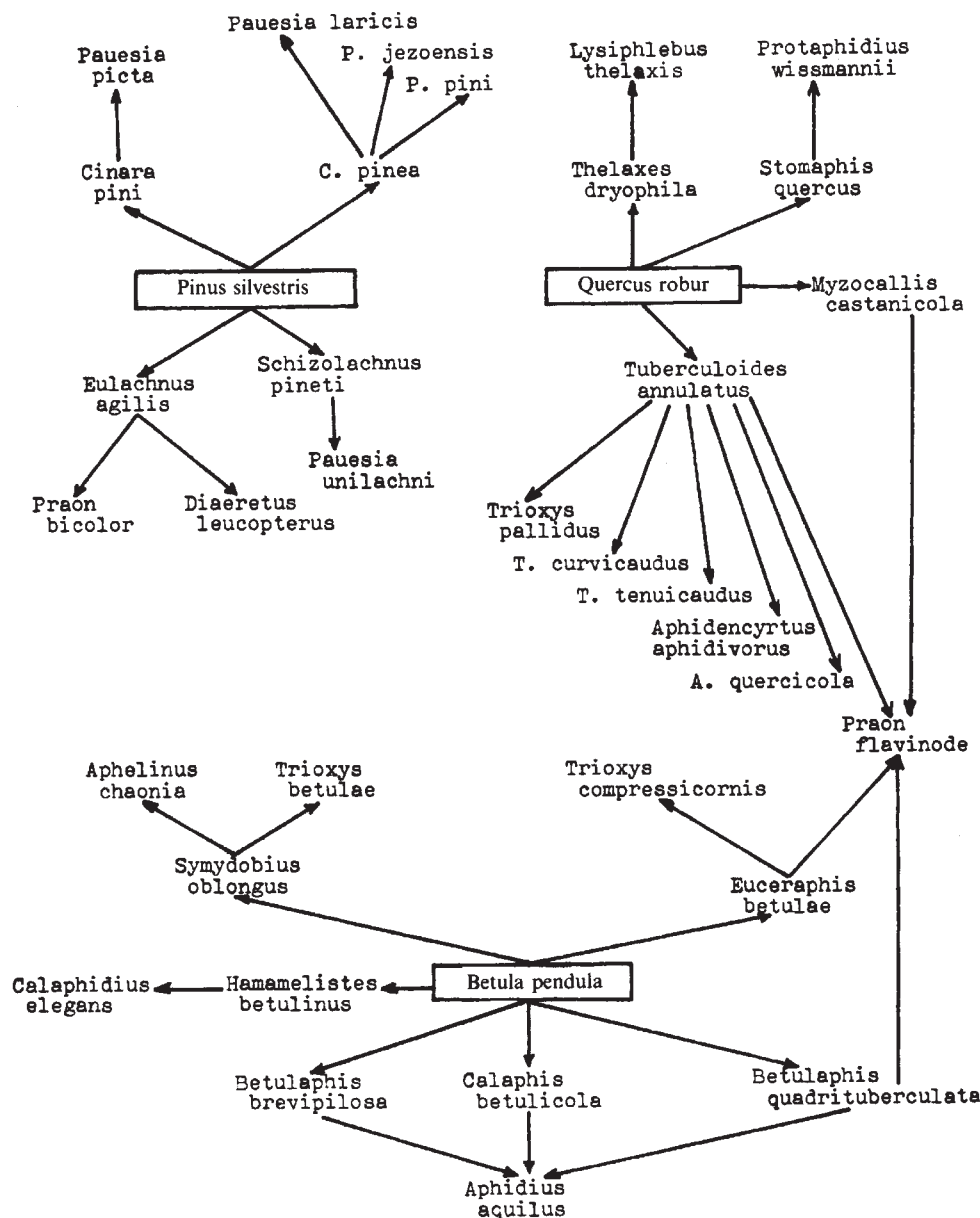


Fig. 1 An example of the plant-aphid-parasitoid food web: a canopy of oak-pine-birch forest in Central Europe (number of species = 37, trophic connectance = 0.059, potential total connectance = 0.137).

unilachni. Other chains are not so remarkable, for example, that of *Betula pendula*–*Betulaphis brevipilosa*–*Aphidius aquilus*. The potential total connectance is highly overestimated because it has been calculated on the basis of both the discovered trophic connections and all potential competitive interactions derived from the structure of the food webs regardless of the biological reality. The aggregation of the populations¹⁸ and the differentiation of the food niches considerably reduce the number of competitive interactions. For example, *Cinara pinea* can never directly compete with *Eulachnus agilis* or *Schizolachnus pineti* despite the common plant host.

Food webs have been reconstructed for 31 plant-aphid-parasitoid communities; the summary of the connectance analysis is presented in Fig. 2. The analysed communities cover a broad variety of Central European biotopes and successional stages. With regard to the previous comments, we may conclude that the decrease in total connectance with increased species richness fits very closely the relationship $C_m = 3$. This reduced connectance ensures the stability of model ecosystems if the standard deviation s of the interaction coefficients is less than 0.577, that is, the variance is less than one-third. It means that with this modest value of s , an increasing number of species does not cause a decrease of model stability: a conclusion which, at

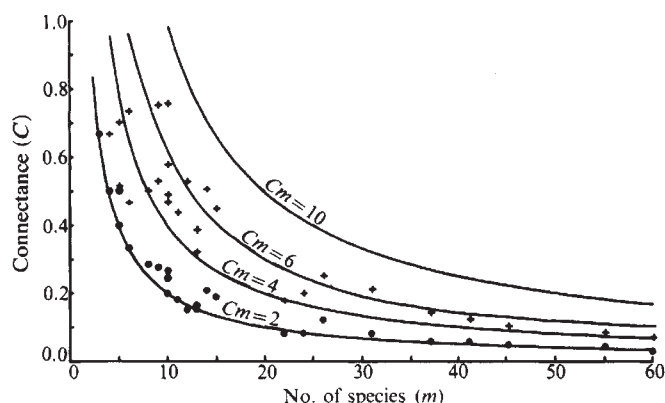


Fig. 2 Four hyperbolic approximations and the empirical relationships between connectance (c) and species number (m) in real plant-aphid-parasitoid communities. Potential total C (+) was calculated on the basis of both the discovered trophic connectance (●) and all potential competitive interactions derived from the structure of the food webs (see, for example, Fig. 1) regardless of the biological reality.

least, does not contradict the traditional ecological hypothesis. Connectance values for pure competition communities have recently been estimated by McNaughton¹⁹. His data for communities consisting of up to 20 plant species follow roughly the product $Cm = 4.7$.

The low connectance assessed by us in plant-aphid-parasitoid communities, arises undoubtedly from their organisation in guilds of species interacting within each guild, whereas their interactions with species in other guilds are only loose. Such an organisation may reduce the destabilising effect of greater system complexity^{1,2}. It seems likely that the majority of the plant-aphid-parasitoid communities possesses structures which correspond to the boundary conditions of model stability. This is, of course, a rather speculative conclusion as we are unable to estimate the s values. On the other hand, there is no evidence for the existence of any plant-aphid-parasitoid communities outside the region $2 < Cm < 6$. Destabilising effects can be expected, through increased connectance caused by hyperparasitoids and predators, both with broader food spectra^{20,21}; further study is required on this point.

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1. May, R. M. *Nature* **238**, 413–414 (1972).
2. May, R. M. *Stability and Complexity in Model Ecosystems* (Princeton University Press, Princeton, 1973).
3. Odum, E. P. *Fundamentals of Ecology* (Saunders, Philadelphia, 1971).
4. May, R. M. in *Theoretical Ecology: Principles and Applications* (ed. May, R. M.) 142–162 (Blackwell, Oxford, 1976).
5. May, R. M. in *The Breakdown and Restoration of Ecosystems* (ed. Holgate, M. W. & Woodman, M. J.) 11–25 (Plenum, New York, 1978).
6. Gilpin, M. E. *Nature* **254**, 137–139 (1975).
7. Goodman, D. Q. *Rev. Biol.* **50**, 237–266 (1975).
8. Pielou, E. C. *Ecological Diversity* (Wiley, New York, 1975).
9. Turk, J., Wittes, J. L., Wittes, R. & Turk, A. *Ecosystems, Energy, Population* (Saunders, Philadelphia, 1975).
10. Whittaker, R. H. *Communities and Ecosystems* 2nd edn (Macmillan, New York, 1975).
11. Goh, B. S. & Jenkins, L. S. *Ecol. Model.* **3**, 63–71 (1977).
12. Logofet, D. O. & Svirežev, Ju. M. *Problemy Kibernetiki* **32**, 187–202 (1977).
13. McLean, J. M., Shepherd, P. & Curnow, R. C. in *New Trends in Mathematical Modelling* (ed. Straszak, A. & Owsinski, J. W.) 257–296 (Ossolineum, Wrocław, 1978).
14. Barnett, S. & Storey, C. *Matrix Methods in Stability Theory* (Nelson, London, 1970).
15. Lawlor, L. R. *Am. Nat.* **112**, 445–447 (1978).
16. Stary, P. *Biology of Aphid Parasites (Hymenoptera: Aphidiidae) with Respect to Integrated Control* (Junk, The Hague, 1970).
17. Stary, P. *Acta Ent. Bohemoslov.* **75**, 164–177 (1978).
18. Way, M. J. & Cammel, M. E. in *Animal Populations in Relation to Their Food Resources* (ed. Watson, A.) 229–247 (Blackwell, Oxford, 1970).
19. McNaughton, S. J. *Nature* **274**, 251–253 (1978).
20. Stary, P. *Acta Ent. Bohemoslov.* **74**, 1–9 (1977).
21. Hodek, I. *Biology of Coccinellidae* (Junk, The Hague, 1973).

Abnormal visual resolution of cats reared in stroboscopic illumination

REARING animals in a visually restricted environment can lead to marked abnormalities of both the response properties of single visual neurones^{1–4} and the visual capacities of the organism^{5,6}. These findings have important implications for understanding the development of human vision and have led to many studies of visually deprived humans^{7–9}. Although there are similarities between the effects of deprivation in animals and humans there are still major difficulties in extrapolating from experimental deprivation to clinically observed amblyopia. There is, therefore, a need for quantitative psychophysical studies of visually deprived animals using the same tests as those used with human patients. We have quantified the pattern vision of visually deprived cats by measuring their spatial contrast

sensitivity, an approach which has frequently been used to characterise the vision of human amblyopes^{7–9}. We report here that the spatial resolution of cats reared in stroboscopic illumination is seriously impaired and that changes in their contrast sensitivity are similar to those seen in certain types of amblyopia.

Stroboscopic illumination prevents the perception of motion while allowing rather restricted pattern vision. Animals reared in this illumination show greatly reduced directional selectivity and binocularity of visual neurones in the superior colliculus and visual cortex^{10,11}, impaired orientation tuning in cortical neurones¹¹, and marked difficulties in visually guided behaviour^{12,13}. However, there is as yet no quantitative description of their visual capacities.

Six cats were used. Three were reared from birth in a room illuminated by a 3- μ s strobe light, flashed at a frequency of 40 min⁻¹ for 12 h per day. One control cat was reared in a long duration (750 ms) flash of the same frequency as the strobe light. This 750-ms flash permitted the perception of motion while providing a control for the periodicity and low luminance of the strobe environment. The effective luminance of these two types of illumination was equated using a behavioural test in which luminance thresholds for grating discrimination were determined in normal cats for each of these light sources (T.P. and W.H.M., in preparation). When the deprived cats were at least a year old they were removed from the visually restricted environment. Testing began after at least 2 months of adaptation to normal illumination. Two more cats were obtained from a supplier and presumably had normal visual experience during development.

Our behavioural testing techniques resembled those developed by Berkley¹⁴. The cats were trained to discriminate between a vertical grating of sinusoidal horizontal luminance profile and a uniform field, each at a mean luminance of 15.9 cd m⁻². They responded by pressing with their nose on one of two transparent response panels through which the stimuli were visible. The stimuli were 30 cm behind the response panels, subtended a visual angle of 16° × 20°, and were generated on display oscilloscopes by conventional techniques¹⁵.

On each trial the stimulus was present for 2 s before the response panel could be activated. Correct responses were rewarded with beef purée and the next trial began 5 s later. Following incorrect responses, no food reward was delivered and the start of the inter-trial interval was delayed 10 s. The position of the correct stimulus varied randomly.

Once the cats mastered the task (4 consecutive days ≥80% correct, 200 trials a day), measurement of contrast thresholds over a range of spatial frequencies began. Performance at a single spatial frequency was tested during each daily session of 200 trials. We used the method of constant stimuli to generate a psychometric function in each session: five contrast levels chosen to bracket the threshold were presented in random order within each block of five trials. Contrast threshold was defined from the resulting psychometric function as the contrast corresponding to 75% correct.

The results are shown in Fig. 1a. At the frequency of peak sensitivity, normal cats and the 750-ms control cat can resolve gratings of ~1.4% contrast. Their sensitivity decreases at lower and higher spatial frequencies. The functions for the three strobe cats show greatly reduced sensitivity at all spatial frequencies and a peak shifted towards lower frequencies. The minimal resolvable contrast for the three cats ranged from 7.6 to 29%. Strobe cats also showed a reduction of about 1.5 octaves in the high-frequency cut-off. Figure 1b shows log sensitivity loss for the three strobe cats, relative to the 750-ms control. This plot clearly demonstrates increasing sensitivity loss with higher spatial frequencies.

The functions in Fig. 1a have shown little change over more than 2 years of testing despite continuing exposure of the cats to a normal laboratory environment. Thus, these deficits are relatively permanent. It is unlikely, moreover, that optical factors can account for these losses. We tested all cats at a distance of 30 cm, which is close to the near point of accommodation for

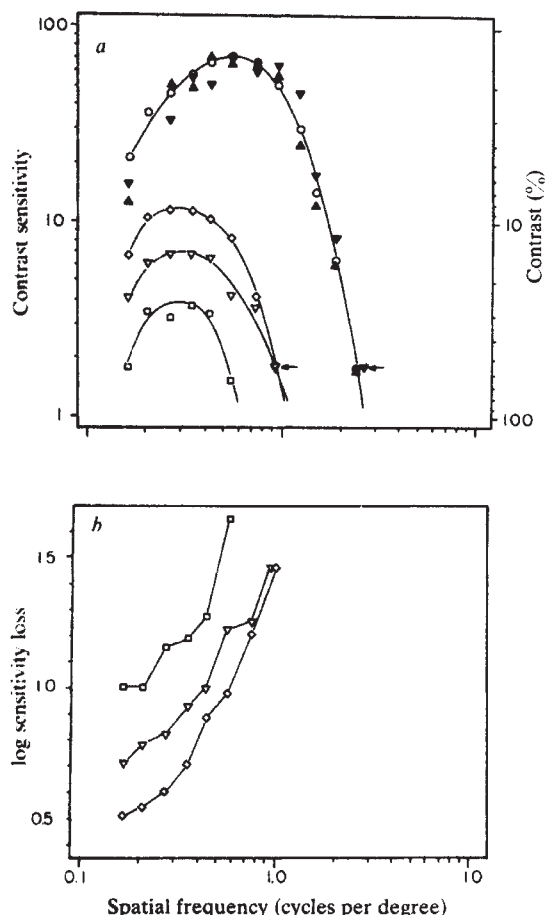


Fig. 1 *a*, Contrast sensitivity (reciprocal of threshold contrast which is shown on the right ordinate) as a function of spatial frequency. $\circ$, 750-ms Control cat; $\triangle$, $\square$, $\diamond$, three strobe-reared cats. Individual curves were fitted by eye. $\blacktriangle$, $\blacktriangledown$, The two normal cats. Points indicated by the arrows are acuity measurements obtained by presenting a range of spatial frequencies at 55% contrast. Each point represents the reciprocal of the contrast value corresponding to 75% correct performance at that frequency and is based on at least 400 trials. Luminance, 15.9 cd m^{-2} ; viewing distance, 30 cm; field size, $20^\circ \times 16^\circ$. *b*, Sensitivity loss for the three strobe-reared cats compared to the 750-ms control cat as a function of spatial frequency. The same symbol is used for each cat as in *a*. Each point represents the ratio of sensitivity of the 750-ms control cat to the sensitivity of the strobe-reared cat expressed in log units. 0.0 corresponds to no loss, 1.0 to a 10-fold reduction in sensitivity.

cats^{1b}. Refraction by split lamp retinoscopy showed our strobe cats to be slightly myopic (-0.5 to -1.5 dioptre), thus if their accommodative power was normal, they should have been able to focus at that distance. Moreover, optical blurring primarily degrades the resolution of high spatial frequencies⁷ and could not account for the losses we observed at low frequencies. Therefore, we suggest that these deficits have a neural basis. Abnormalities in area 17, which have been reported in strobe-reared cats¹¹, could reduce behavioural contrast sensitivity as striate neurones are particularly sensitive to high spatial frequencies and have low contrast thresholds¹⁷.

The profound visual loss of strobe-reared cats indicates that strobeoscopic illumination, which has been used to examine the effects of motion deprivation, also severely restricts spatial vision. However, the normal contrast sensitivity of the cat reared in the long-duration flash demonstrates that the visual loss of strobe-reared cats is not due to either the low time-averaged luminance or periodicity of the strobe illumination. The pattern

of visual loss, with the greatest impairment at high spatial frequencies, is similar to that found in strabismic^{8,18} and anisometric⁸ amblyopia, suggesting common features between the visual environments of strobe-reared cats and human amblyopes. The visual deficit in these types of amblyopia has been attributed to the consistently blurred image in the amblyopic eye, which has its greatest impact on the visibility of high spatial frequencies. Optical blur may also be an important factor in strobe rearing as the extremely short-flash duration precludes normal accommodation, thus limiting the visibility of high spatial frequencies.

A greater loss at high than at low spatial frequencies in human amblyopes is commonly attributed to a selective impairment of sustained visual neurones^{8,9}, which, in contrast to transient neurones, respond better to higher spatial frequencies but prefer slower rates of temporal modulation¹⁹. The contrast sensitivity data reported here as well as the recent finding of reduced sensitivity to slow motion in strobe-reared cats²⁰ suggests that the major impact of strobe rearing is also on the sustained neurones. Consistent with this interpretation is the observation that sustained neurones are especially dependent on proper accommodation²¹ and that they project exclusively to area 17 (ref. 22), which is highly abnormal in strobe-reared animals. Future measurements of the contrast sensitivity of cortical neurones in these cats will help clarify the relationship between neural and perceptual abnormalities in strobe rearing and may provide important insights into neural mechanisms involved in high spatial frequency loss.

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- Wiesel, T. N. & Hubel, D. H. *J. Neurophysiol.* **26**, 978-993 (1963); *J. Neurophysiol.* **26**, 1003-1017 (1963); *J. Neurophysiol.* **28**, 1029-1040 (1965).
- Blakemore, C. & Cooper, G. F. *Nature* **228**, 477-478 (1970).
- Pettigrew, J. D. & Freeman, R. D. *Science* **182**, 599-601 (1973).
- Cynader, M., Berman, N. & Hein, A. *Expl Brain Res.* **22**, 267-280 (1975).
- Dews, P. B. & Wiesel, T. N. *J. Physiol., Lond.* **206**, 437-455 (1970).
- Muir, D. W. & Mitchell, D. E. *Brain Res.* **85**, 459-477 (1975).
- Levi, M. & Harwerth, R. S. *Invest. Ophthalm. vis. Sci.* **16**, 90-94 (1977).
- Hess, R. F. & Howell, E. R. *Vision Res.* **17**, 1049-1055 (1977).
- Wood, I. C. J. & Kulikowski, J. J. *Vision Res.* **18**, 331-334 (1978).
- Flandrin, J. M., Kennedy, H. & Amblard, B. *Brain Res.* **101**, 576-581 (1976).
- Cynader, M., Berman, N. & Hein, A. *Expl Brain Res.* **25**, 139-156 (1976).
- Orbach, J. & Miller, M. H. *Vision Res.* **9**, 713-716 (1969).
- Hein, A., Gower, B. C. & Diamond, R. M. *J. comp. Physiol. Psychol.* **73**, 188-192 (1970).
- Pasternak, T., Merigan, W. H. & Dardano, R. *Invest. Ophthalm. vis. Sci. Suppl.* **148** (1979).
- Ikeda, H. & Wright, M. J. *Vision Res.* **12**, 1465-1476 (1972).
- Stone, J. & Dreher, B. *J. Neurophysiol.* **36**, 551-567 (1973).

An intrinsic factor XII-prekallikrein-dependent pathway activates the human plasma renin-angiotensin system

EXPOSURE of normal human plasma to an activating (negatively charged) surface leads to the activation of the intrinsic coagulation system and the fibrinolytic system and the generation of vasoactive peptides (kinins)^{1,2}. This contact activation mechanism is known to be initiated by factor XII (Hageman factor). Surface-activated factor XII converts prekallikrein (Fletcher factor) into kallikrein, which leads to further activation of factor XII. Prekallikrein circulates in plasma as a complex with the kallikrein substrate, high molecular weight kininogen, and the latter acts as a cofactor in the reciprocal activation of factor XII by kallikrein. The experiments described here demonstrate that the renin-angiotensin system is also activated by kallikrein through the factor XII-dependent pathway.

Normally, about 80% of the renin present in plasma is in an enzymatically inactive form³⁻⁵. It is called 'inactive renin' or 'prorenin', as opposed to naturally occurring 'active renin'. Both forms can be separated by ion-exchange chromatography⁶. Inactive renin is converted into the active form by limited proteolysis at neutral pH, either in acid-pretreated plasma by one or more endogenous serine proteases⁷⁻⁹ and by urinary (renal) kallikrein¹⁰, or in untreated plasma with exogenous trypsin^{5,9}. Active renin is also formed during prolonged exposure of plasma to low temperature (cryoactivation)¹¹. Recent reports have suggested that factor XII and prekallikrein might be involved in the generation of active renin, but the results were contradictory^{12,13}. To investigate the possible involvement of the factor XII-dependent pathway in the activation of prorenin, we treated normal plasma ($n = 10$), factor XII-deficient plasma ($n = 2$) and prekallikrein-deficient plasma ($n = 1$) by (1) dialysis at pH 3.3 and 4 °C for 24 h with subsequent restoration of pH to 7.5 (ref. 14), or (2) incubation with Sepharose-bound trypsin at pH 7.5 and 4 °C. Renin was then measured by radioimmunoassay of angiotensin I formed after incubation of the plasma samples with an excess of purified sheep renin substrate at pH 7.5 and 37 °C for 3 h (ref. 4). Results in normal plasma showed identical amounts of activated renin after acid or trypsin treatment (Fig. 1, left-hand scale). Smaller increments of active renin were obtained during cryoactivation. In plasma from patients with Hageman trait, levels of naturally occurring active renin were 17 and 21 $\mu\text{U ml}^{-1}$, and those of inactive (trypsin-activable) renin 180 and 210 $\mu\text{U ml}^{-1}$. These values were within the normal range found in our laboratory, which is 12–45 $\mu\text{U ml}^{-1}$ for active renin and 70–230 $\mu\text{U ml}^{-1}$ for inactive renin ($n = 25$). In samples from the patient with Fletcher trait, active renin was low, 8.4 $\mu\text{U ml}^{-1}$, but inactive renin was markedly elevated, 510 $\mu\text{U ml}^{-1}$. In contrast to normal plasma, both factor XII- and prekallikrein-deficient plasma generated small amounts of active renin after acidification, whereas trypsin generated normal or increased amounts. There was no detectable cryoactivation of renin in the deficient plasmas. This suggested that acid activation and cryoactivation of plasma renin depend on the presence of both factor XII and prekallikrein.

Further experiments substantiated this hypothesis. Reconstitution of factor XII-deficient plasma and prekallikrein-deficient plasma with, respectively, purified factor XII and prekallikrein resulted in a correction of the generation of active renin after acidification. Prekallikrein in normal plasma is converted into active kallikrein after exposure of the plasma to low pH, trypsin or low temperature (cryoactivation)¹⁵. Simultaneous determinations of renin and kallikrein measured as amidolytic activity¹⁶ showed parallel increments of both activities (Fig. 1, right-hand scale).

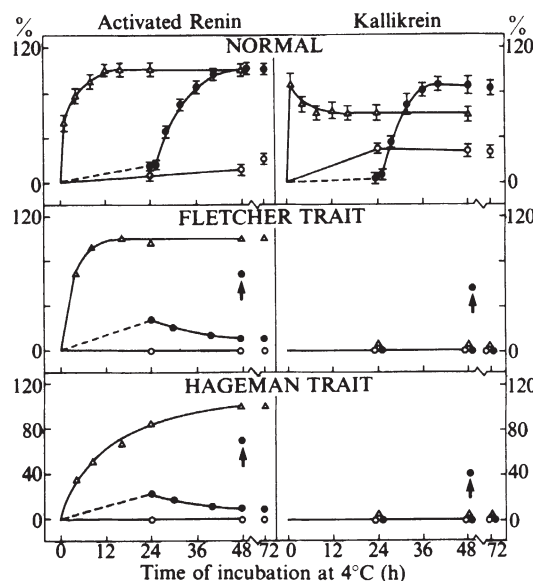


Fig. 1 Generation of active renin and kallikrein in normal plasma (mean $\pm$ s.e.m., $n = 10$), prekallikrein (Fletcher factor)-deficient plasma and factor XII (Hageman factor)-deficient plasma. Two procedures were used: acidification and addition of trypsin. Plasma, which contained 5×10^{-3} M EDTA, was acidified (---○---) by dialysis against 0.15 M glycine-HCl buffer of pH 3.3 for 24 h (ref. 14). The pH was then restored to 7.5 with 1 M NaOH, and the generation of active renin and kallikrein was followed for 1–72 h (●—●). Trypsin (14,000 BAEE units per mg protein, Sigma) was covalently bound to CNBr-activated Sepharose (Pharmacia) in a ratio of 10 mg protein per g dry Sepharose. The immobilised enzyme was added to plasma (Δ — Δ) at a final concentration of 3,000 BAEE units per ml. After 1–48 h it was removed by centrifugation. All these procedures were carried out at 4 °C. Plasma that was kept at 4 °C without any other form of treatment served as a control (○—○). Pretreated and control plasma samples were then assayed for renin and kallikrein activity. For renin measurements the samples were incubated with purified sheep renin substrate (final concentration $6.7 \times K_m$)⁴ at pH 7.5 and 37 °C for 3 h in the presence of the following protease (angiotensinase) inhibitors: 1×10^{-3} M EDTA, 1×10^{-3} M phenylmethylsulphonylfluoride, 3.4×10^{-3} M 8-hydroxyquinoline and aprotinin (Bayer) at a concentration of 100 kallikrein-inhibiting units per ml of incubate. The increment of renin after 48 h exposure to trypsin was taken as 100% activation. Kallikrein was measured as amidolytic activity using the chromogenic ester H-D-Pro-Phe-Arg-p-nitroanilide (PPAN, Kabi) as a substrate¹⁶. Plasma (15 μl) was mixed at 37 °C with 815 μl Tris buffer of pH 7.5 and 200 μl 4.1×10^{-3} M PPAN. The linear release of p-nitroanilide was followed at 405 nm for 1–2 min in a 1-cm semi-microcuvette at 37 °C. Complete activation of prekallikrein was obtained by mixing 100 μl of the untreated plasma with 100 μl dextran sulphate (25 mg l^{-1})¹⁸. After 7 min incubation at 4 °C, 30 μl of the mixture was added to 800 μl Tris buffer and 200 μl PPAN, and kallikrein was measured as before. The values obtained after dialysis at pH 3.3 and restoration of pH to 7.5 and with trypsin were expressed as a percentage of total activable prekallikrein as obtained with dextran sulphate. Lineweaver-Burk plots for the reactions of acid-activated renin, trypsin-activated renin and naturally occurring active renin with sheep renin substrate (1 h incubation) gave identical K_m values ($\sim 6 \times 10^{-7}$ mol l^{-1}). Thus, the increase of renin activity after acidification and with trypsin is caused by a parallel change in the number of molecules of active renin rather than by changes in the enzymatic activity per molecule of active renin. When untreated normal plasma was kept at 4 °C, small increments in renin and kallikrein activity were observed ($P < 0.001$ for difference of values at 72 h from those at 0 h). Prekallikrein and factor XII were isolated from normal human plasma and purified¹⁹. They were added to, respectively, prekallikrein-deficient plasma (final concentration of prekallikrein $\sim 50 \mu\text{g ml}^{-1}$) and factor XII-deficient plasma (final concentration of factor XII $\sim 28 \mu\text{g ml}^{-1}$). Deficient plasmas to which Tris buffer of pH 7.5 was added, instead of prekallikrein or factor XII, served as controls. The reconstituted plasmas and controls were treated by dialysis at pH 3.3 for 24 h, and the renin and kallikrein activities were measured 24 h after the pH had been restored to 7.5 (results are indicated by arrows).

The acid activation of prekallikrein was confirmed by the parallel cleavage of ¹²⁵I-labelled prekallikrein added to normal plasma before acidification (Fig. 2). Previous experiments in which the activation of prekallikrein by activated factor XII was studied had shown that the generation of kallikrein activity was directly correlated with the extent of proteolytic cleavage¹⁷. Analysis of the reaction mixture by SDS-polyacrylamide gel electrophoresis in the presence of reducing agents demonstrated that the disappearance of prekallikrein of apparent molecular

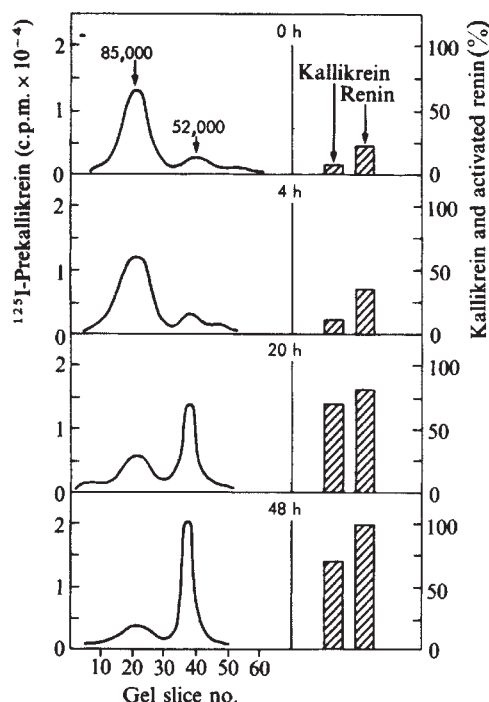


Fig. 2 Cleavage of ^{125}I -prekallikrein in acid-pretreated normal plasma. Prekallikrein was isolated from normal plasma¹⁹ and labelled by the method of Bolton and Hunter²⁰. ^{125}I -prekallikrein ($\sim 2.7 \mu\text{Ci}$ in $15 \mu\text{l}$) was added to $600 \mu\text{l}$ normal plasma. The mixture was dialysed at pH 3.3 for 24 h. Immediately (0 h) after restoration of pH to 7.5 with 1 M NaOH, and 4–48 h later, the reaction mixtures were analysed for the presence of cleavage products of ^{125}I -prekallikrein (left) and for kallikrein and renin activity (right). After reduction by β -mercaptoethanol the reaction mixture was subjected to SDS-polyacrylamide gel (7.5%) electrophoresis. The gels were sliced and the radioactivity in each slice (1.2 mm) was counted. The appearance of cleavage products with an apparent molecular weight of 52,000 indicates activation of prekallikrein¹⁷.

weight 85,000 was associated with the simultaneous generation of cleavage fragments; the major radiolabelled fragment had an apparent molecular weight of 52,000.

Addition of a physiological amount of purified plasma kallikrein to factor XII-deficient plasma after the acidification step, caused complete activation of inactive renin (Fig. 3).

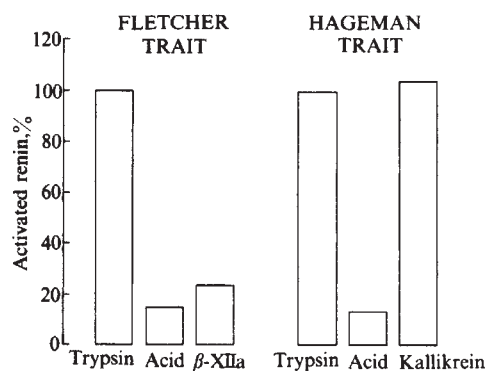


Fig. 3 Generation of active renin in prekallikrein (Fletcher factor)-deficient plasma and factor XII (Hageman factor)-deficient plasma. Plasma was treated with Sepharose-bound trypsin at pH 7.5 and 4°C for 48 h or was dialysed at pH 3.3 for 24 h followed by dialysis at pH 7.5 and 4°C for 24 h. Factor β -XIIa (final concentration $10 \mu\text{g ml}^{-1}$) was added to prekallikrein-deficient plasma after 24 h dialysis of the plasma at pH 3.3 and restoration of pH to 7.5. Plasma kallikrein (final concentration $32 \mu\text{g ml}^{-1}$) was added to factor XII-deficient plasma again after 24 h dialysis of the plasma at pH 3.3 and restoration of pH to 7.5. The mixtures were incubated at 4°C for 24 h. Factor β -XIIa ($600 \mu\text{g ml}^{-1}$) (from Dr John Griffin) gave a single band on SDS-polyacrylamide gel electrophoresis with an apparent MW of 28,000. Kallikrein ($200 \mu\text{g ml}^{-1}$) was prepared by activation of plasma prekallikrein by factor β -XIIa and was separated from factor β -XIIa by DEAE-Sephadex chromatography at pH 8.25¹⁹.

Active factor XII fragment (β -XIIa) in a physiological concentration, however, was not capable of generating active renin in prekallikrein-deficient plasma. This indicates that in the factor XII-dependent pathway, plasma kallikrein is the major activator of inactive renin. The factor XII-dependency can be explained by the capacity of this factor to convert plasma prekallikrein into kallikrein.

We conclude that factor XII and prekallikrein are part of an endogenous pathway that activates renin *in vitro*. It is of interest that inactive (trypsin-activable) plasma renin was grossly elevated in the patient with Fletcher trait, whereas his plasma level of naturally occurring active renin was abnormally low. This may indicate that the factor XII-prekallikrein pathway is also important for the activation of renin *in vivo*. Possibly, inactive renin that is adsorbed or bound to the blood vessel wall, at sites that are inaccessible to circulating protease inhibitors, is activated through this pathway. The reported activation of prorenin after the addition of renal kallikrein to acid-pretreated plasma¹⁰ suggests a further analogy with the clotting and fibrinolytic systems: plasma kallikrein is part of an intrinsic pathway for activating the renin-angiotensin system, whereas tissue (renal) kallikrein might be involved in extrinsic activation.

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- Kaplan, A. P. & Austen, K. F. *J. Allergy clin. Immun.* **56**, 491–506 (1975).
- Ogston, D. & Bennett, B. *Br. med. Bull.* **34**, 107–112 (1978).
- Skinner, S. L. *et al. Am. J. Obstet. Gynec.* **121**, 626–630 (1975).
- Derckx, F. H. M. *et al. Lancet* **ii**, 496–499 (1976).
- Leckie, B. J. *et al. Circ. Res.* **40**, Suppl. 1, 46–51 (1977).
- Shulkes, A. A., Gibson, R. R. & Skinner, S. L. *Clin. Sci.* **55**, 41–50 (1978).
- Morris, B. J. & Lumbers, E. R. *Biochim. biophys. Acta* **289**, 385–391 (1972).
- Derckx, F. H. M., Tan-Tjong, H. L. & Schalekamp, M. A. D. H. *Lancet* **ii**, 218–219 (1978).
- Cooper, R. M., Murray, G. E. & Osmond, D. H. *Circ. Res.* **40**, Suppl. 1, 171–179 (1977).
- Sealey, J. E., Atlas, S. A., Laragh, J. H., Oza, N. B. & Ryan, J. W. *Nature* **275**, 144–145 (1978).
- Osmond, D. H., Ross, L. J. & Scaiff, K. D. *Can. J. Physiol. Pharmac.* **51**, 705–708 (1973).
- Osmond, D. H., Lo, E. K., Loh, A. Y., Zingg, E. A. & Hedlin, A. H. *Lancet* **ii**, 1375–1376 (1978).
- Millar, J. A., Clappison, B. H. & Johnston, C. I. *Lancet* **ii**, 1376 (1978).
- Skinner, S. L. *Circ. Res.* **20**, 392–402 (1967).
- Eisen, V. & Vogt, W. in *Handbook of Experimental Pharmacology* (ed. Erdös, E. G.) 82–130 (Springer, New York, 1970).
- Claesson, G., Friberger, P., Knös, M. & Eriksson, E. *Haemostasis* **7**, 76–79 (1978).
- Bouma, B. N., Miles, L. & Griffin, J. H. *J. biol. Chem.* (in the press).
- Kluft, C. *J. Lab. clin. Med.* **91**, 83–95 (1978).
- Griffin, J. H. & Cochrane, C. G. *Meih. Enzym.* **45**, 56–65 (1976).
- Bolton, A. E. & Hunter, W. M. *Biochem. J.* **133**, 529–539 (1973).

Macrophage requirements for mouse B-cell stimulation differ according to the molecular form of anti-IgM antibodies

THYMUS-INDEPENDENT lymphocytes (B cells) comprise a heterogeneous population. Because of this heterogeneity, the precise nature of the signals which activate resting B cells to become antibody-forming cells is not known. It is likely that different subsets of B cells have different signalling requirements^{1–3}. In addition, it has been proposed that B-cell proliferation and differentiation are controlled by separate and distinct signals⁴. Proliferation is thought to result from antigen binding with specific cell-surface immunoglobulin (Ig) receptors. However, due to the very low frequency of B cells reactive to a specific antigen, the activation process *per se* is difficult to study. This problem may be overcome by using reagents which mimic specific antigen binding to Ig receptors but act as poly-

Table 1 Proliferative response of spleen cells to various stimulants

Stimulant	Dose (μ g per well)	Cells cultured (c.p.m. $\pm$ s.e.m.)					
		Unseparated spleen cells	0%*	Macrophage-depleted spleen cells Peritoneal macrophages added			
				2%	4%	8%	16%
NRG	100	1,710 $\pm$ 90	370 $\pm$ 30	1,350 $\pm$ 29	1,320 $\pm$ 95	1,210 $\pm$ 113	1,440 $\pm$ 81
Anti-IgM	100	6,650 $\pm$ 1,430	510 $\pm$ 38	12,850 $\pm$ 1,473	7,320 $\pm$ 50	5,242 $\pm$ 369	1,710 $\pm$ 145
F(ab') ₂ NRG	20	2,700 $\pm$ 125	1,100 $\pm$ 53	2,380 $\pm$ 80	2,760 $\pm$ 171	3,120 $\pm$ 254	2,890 $\pm$ 599
F(ab') ₂ anti-IgM	20	45,000 $\pm$ 2,948	42,650 $\pm$ 2,065	59,330 $\pm$ 1,675	65,370 $\pm$ 2,339	51,790 $\pm$ 1,159	25,560 $\pm$ 765
Control	—	3,500 $\pm$ 368	640 $\pm$ 148	1,920 $\pm$ 59	3,510 $\pm$ 140	3,240 $\pm$ 466	2,820 $\pm$ 499
LPS	1	66,020 $\pm$ 4,459	51,650 $\pm$ 2,065	30,200 $\pm$ 872	32,400 $\pm$ 838	21,440 $\pm$ 495	11,570 $\pm$ 684

Unseparated or macrophage-depleted spleen cells from 8-month-old CBA mice were cultured in triplicate for 3 d in flat-bottom microtitre plates at 4×10^5 cells per well. The cells were stimulated by addition of optimal doses of rabbit anti-IgM, F(ab')₂ anti-IgM or *Escherichia coli* 011B4 LPS. Control cultures received normal rabbit globulin (NRG), F(ab')₂ NRG or medium. Normal peritoneal macrophages were collected by lavage from CBA mice and added to macrophage-depleted cultures as indicated. All cultures were pulsed with 0.5 μ Ci of ³H-thymidine for the last 24 h of culture. ³H-thymidine incorporation is expressed as mean c.p.m. of triplicate cultures $\pm$ s.e.m.

* Macrophage-depleted spleen cells contained <1% macrophages as measured by esterase staining.

clonal B-cell activators (PBAs). Anti-Ig antibodies are thought to function in this way and have been shown to be mitogenic for B cells⁵⁻⁷. Our laboratory has previously shown that soluble rabbit anti-mouse IgM or F(ab')₂ anti-IgM is mitogenic for mouse splenic B cells^{8,9}. The response to intact anti-IgM only occurs with B cells from mice which are 7 months or older. In contrast, F(ab')₂ anti-IgM will stimulate B cells from both young and aged mice equally well suggesting that changes in B-cell Fc receptors may be involved in the age-dependent response to the intact anti-IgM molecule¹⁰. In all cases, the response requires cross-linkage of surface μ heavy chains and is T-cell independent^{10,11}. In the present study, we have investigated the macrophage requirement for anti-Ig stimulation. The requirement for macrophages in B-cell activation by PBAs, including anti-Ig reagents, is controversial^{12,13}. We report that the proliferative response of B cells to intact anti-IgM is macrophage dependent whereas the response of the same cell population to F(ab')₂ anti-IgM is macrophage independent.

Spleen cells and peritoneal cells were prepared from 8–10-month-old CBA/J mice. Peritoneal cells were obtained by lavage of the cavity with cold balanced salt solution containing 10% fetal calf serum (BSS-FCS). This procedure yielded 7–8 $\times 10^6$ peritoneal cells per mouse of which at least 60% were macrophages as judged by esterase staining and which are hereafter referred to as peritoneal macrophages. When these cells were added to macrophage-depleted spleen cell cultures, the cell number was adjusted to compensate for non-macrophages. Spleen cells were prepared by gently pressing the tissue through a 60-mesh wire screen into cold BSS-FCS followed by two washes. Macrophages were depleted by a two-step procedure. First, the spleen cells were incubated for 90 min in BSS-FCS at 37 °C in plastic Petri dishes. Next, the non-adherent cells (2×10^8) were passed slowly through a Sephadex G-10 column¹⁶ (18 cm³ of packed Sephadex in a 20-cm³ syringe) at 37 °C and eluted with warm BSS-FCS. Cells eluting in the first 5 ml were collected for culture. In several replicate experiments, this population contained 50–65% B cells and less than 1% macrophages as judged by immunofluorescent staining and esterase staining, respectively. Less than 0.1% of the G-10-passed cells were phagocytic for latex beads. Conditions for culture and stimulation with intact anti-IgM (anti-MC774 IgM myeloma)⁸, F(ab')₂ anti-IgM¹¹ or lipopolysaccharide (LPS) are given in Table 1.

As shown in Table 1, unseparated spleen cells responded to all three stimulants. Macrophage-depleted spleen cells, however, failed to respond to intact anti-IgM but responded normally to F(ab')₂ anti-IgM and LPS. The lack of response to intact anti-IgM was not dose dependent; the cells remained unresponsive when cultured with a wide range of doses of anti-IgM (not shown). Addition of peritoneal macrophages (2%) to the macrophage-depleted spleen cell cultures completely restored,

and actually enhanced, the response to intact anti-IgM. The response to F(ab')₂ anti-IgM was slightly increased whereas the response to LPS was somewhat inhibited. (Macrophages cultured alone at four different cell concentrations do not respond to any of the stimulants (data not shown).) Addition of increasing numbers of macrophages to the cultures (4% and 8%) reduced the response to intact anti-IgM to a level approximately equal to the response of unseparated spleen cells. At the same time, the response to F(ab')₂ anti-Ig remained relatively unchanged whereas the response to LPS was markedly inhibited. Responses to all three stimulants were significantly inhibited when 16% macrophages were added to the cultures. These results indicate that the macrophage requirements for stimulating B-cell proliferation with different anti-Ig reagents differ and depend, at least in part, on the molecular species of anti-Ig antibody used.

Mongini *et al.* have reported that the *in vitro* proliferative response of mouse B cells to soluble goat anti-IgM is macrophage dependent¹⁵. Our results confirm their findings, and we have extended the data to show that the response to F(ab')₂ anti-IgM is macrophage independent. In contrast, Sieckmann *et al.* have reported that intact anti-IgM stimulation of mouse B cells is macrophage independent¹⁴. However, as their culture media contained 2-mercaptoethanol (2-ME), that interpretation is not completely valid (see below).

It is not clear exactly why B cells require macrophages to respond to intact anti-IgM molecules but do not require accessory cells to respond to the F(ab')₂ form of the same molecule. Several possibilities must be considered. Additional experiments (not shown) have shown that 2-ME can substitute for macrophages and completely restore the response of macrophage-depleted spleen cells to intact anti-IgM. 2-ME is generally thought to replace supportive functions of macrophages related to cell viability¹⁷. It is unlikely, however, that the inability of macrophage-depleted cultures to respond to intact anti-IgM is due to problems of B-cell viability, as these same cultures respond normally to LPS and F(ab')₂ anti-IgM. Rather, 2-ME may simply potentiate the effect of the few remaining macrophages which are then able to support the induction of anti-IgM stimulation. This may involve the secondary or 'helper' signals provided by the macrophages. Alternatively, 2-ME may act independently and generate a cofactor from the serum which acts directly on the B cells¹⁸. Whatever the case, these secondary signals seem not to be required for stimulation by F(ab')₂ anti-IgM. A role for macrophage Fc receptors must also be considered. It is possible that macrophages bind the intact anti-IgM molecules through their Fc receptors and present them to the B cells in an insoluble matrix array. This would be similar to the stimulation of B cells by anti-Ig insolubilized on Sepharose beads as described by Parker¹⁹. However, this seems unlikely, as Mongini *et al.* reported that anti-IgM-pulsed and

washed macrophages could not stimulate macrophage-depleted B cells in the absence of additional soluble anti-IgM¹⁵.

The interpretation we favour is that the differential requirement for macrophages is a reflection of different subsets of B cells responding to the two molecular forms of the anti-IgM antibodies. As these two stimulants differ only with respect to the presence of the Fc portion of the Ig molecules, differential sensitivity of the responding B cells to Fc receptor-mediated negative signals may also be involved. Recent experiments in our laboratory support these interpretations. We have found that complement receptor-positive (CR⁺) B cells from aged mice (> 7 months old) respond only to F(ab')₂ anti-IgM whereas CR⁻ B cells respond preferentially to intact anti-IgM²⁰. The inability of CR⁺ B cells to respond to the intact molecule is not reversed by addition of macrophages or 2-ME. Thus, it seems that intact anti-IgM and F(ab')₂ anti-IgM stimulate different populations of B cells and that these subsets have different signalling requirements for activation. In the context of this report, these findings suggest that depletion of macrophages from whole spleen cell populations selectively eliminates the ability of CR⁻ B cells to respond to intact anti-IgM.

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1. Lemke, H. *et al.* *Scand. J. Immunol.* **4**, 707-720 (1975).
2. Lewis, G. K., Ranken, R., Nitecki, D. E. & Goodman, J. M. *J. Exp. Med.* **144**, 382-397 (1976).
3. Persson, U. C. I., Hammarström, L. L. G. & Smith, C. I. *J. Immunol.* **119**, 1138-1144 (1977).
4. Dutton, R. W. *Transplant. Rev.* **23**, 66-77 (1975).
5. Sell, S. & Gell, P. G. H. *J. exp. Med.* **122**, 423-440 (1965).
6. Skamene, E. & Ivanyi, J. *Nature* **221**, 681-682 (1969).
7. Gausset, P. *et al.* *J. Immunol.* **116**, 446-453 (1976).
8. Weiner, H. L., Moorhead, J. W. & Claman, H. N. *J. Immunol.* **116**, 1656-1661 (1976).
9. Weiner, H. L., Scribner, D. J. & Moorhead, J. W. *J. Immunol.* **120**, 1907-1912 (1978).
10. Scribner, D. J., Weiner, H. L. & Moorhead, J. W. *J. Immunol.* **121**, 377-382 (1978).
11. Weiner, H. L., Moorhead, J. W., Yamaga, K. & Kubo, R. T. *J. Immunol.* **117**, 1527-1531 (1976).
12. Persson, U. *et al.* *Immun. Rev.* **40**, 78-101 (1978).
13. Diener, E. & Lee, K. C. in *Progress in Immunology III* (eds Mandel, T. E. *et al.*) 413-421 (Australian Academy of Science, 1977).
14. Sieckmann, D. G. *et al.* *J. exp. Med.* **148**, 1628-1643 (1978).
15. Mongini, P., Friedman, S. & Wortis, H. *Nature* **276**, 709-711 (1978).
16. Ly, I. A. & Mishell, R. *J. Immun. Meth.* **5**, 239-247 (1974).
17. Chen, C. & Hirsch, J. G. *J. exp. Med.* **136**, 604-717 (1972).
18. Sidman, C. L. & Unanue, E. R. *Proc. natn. Acad. Sci. U.S.A.* **75**, 2401-2405 (1978).
19. Parker, D. C. *Nature* **258**, 361-363 (1975).
20. Schrier, R. D., Hattori, M. & Moorhead, J. W. *J. Immunol.* (in the press).

Table 1 Effect of MNNG on pancreatic acini

	% Discharge (2 h incubation)	Cyclic GMP level (2-min incubation) (fmol per µg DNA)
Experiment 1		
Unstimulated	10.1 ± 2.5	19 ± 2
Caerulein 10 ⁻⁹ M	56.8 ± 1.0	120 ± 37
MNNG 10 ⁻³ M	4.0 ± 2.5	858 ± 77
MNNG 10 ⁻⁴ M	9.1 ± 2.2	1,111 ± 236
MNNG 10 ⁻⁵ M	12.3 ± 1.5	24 ± 2
MNNG 10 ⁻⁶ M	13.4 ± 1.8	11 ± 2
Experiment 2		
Unstimulated	8.8 ± 0.6	40 ± 8
Caerulein 10 ⁻⁹ M	28.9 ± 2.7	317 ± 17
MNNG 10 ⁻⁴ M	11.2 ± 0.7	2,989 ± 694
MNNG 10 ⁻⁵ M	9.2 ± 2.6	363 ± 88
Caerulein 10 ⁻⁹ M + MNNG 10 ⁻⁴ M	28.8 ± 2.9	4,017 ± 751
MNNG 10 ⁻⁴ M added every 30 min	9.9 ± 1.4	NA
Caerulein 10 ⁻⁹ M + MNNG 10 ⁻⁴ M, with MNNG added every 30 min	25.7 ± 4.2	NA

Acini were prepared from pancreata of adult female guinea pigs using purified collagenase and mild mechanical shear^{26,27}. Secretory proteins in acini to be used for measuring protein discharge were first pulse-labelled for 10 min at 37 °C with 4,5-³H-L-leucine (5 µCi ml⁻¹, 40-60 Ci mmol⁻¹, Schwarz/Mann) in 10 ml of a solution (KRH) consisting of Krebs-Ringer's salts, 2% (v/v) essential and 1% non-essential amino acid concentrates (GIBCO), 2 mM L-glutamine, 14 mM D-glucose, 0.2% bovine plasma albumin (Reheis), 0.01% soybean trypsin inhibitor (Worthington) and 24 mM HEPES (Sigma), pH 7.4. Solutions were equilibrated with O₂. Following the pulse, unincorporated ³H-leucine was removed by centrifugation and resuspension of acini in KRH. For determination of either secretory protein discharge or levels of cyclic GMP, acini from 1 g tissue were resuspended in 20 ml KRH containing an additional 3.8 mM unlabelled L-leucine, the substances indicated were added as 100-fold concentrates to 1-ml aliquots of the cell suspension, and incubation at 37 °C was continued for either 2 min (for cyclic GMP) or 2 h (for secretory protein discharge). MNNG was added to acini as a 100-fold concentrate in acetone. Addition of 1% acetone alone had no effect on either discharge or cyclic GMP response. Measurements of cyclic GMP were made on total acinar incubations (cells plus media). To monitor cyclic GMP recovery during purification, each incubation tube received 1.6 nCi ³H-cyclic GMP (15 Ci mmol⁻¹, Schwarz/Mann) at the end of incubation and the reaction was stopped by addition of 0.5 ml 15% trichloroacetic acid (TCA) and freezing. The tubes were then thawed, sonicated for 30 s, centrifuged, and the supernatants passed over cation exchange resin²⁸. To remove TCA, column fractions were extracted five times with eight volumes of water-saturated ether and lyophilised. Samples were redissolved in water and aliquots taken to determine cyclic GMP recovery (range: 40-90%). Cyclic GMP content was determined on the remainder of the sample by radioimmunoassay²⁹ with the modification that the antigen-antibody complex was precipitated with 7% TCA, pH 1.75. MNNG had no effect on antibody binding when added to the radioimmunoassay, and cyclic GMP from acini exposed to MNNG was sensitive to degradation by cyclic nucleotide phosphodiesterase (Boehringer, Mannheim). Total DNA in cell pellets was determined by the method of Burton³⁰. Discharge of labelled secretory proteins was determined as described previously³¹ following separation of acini and media by centrifugation at 800g for 5 min. Results are expressed as percentage discharge, defined as acid-precipitable d.p.m. in the media divided by total d.p.m. (cell pellets plus media) × 100. Values shown are means of triplicate determinations (±s.d.) from individual experiments. With 10⁻⁹ M caerulein, the average net percentage discharge (total minus resting) in 2 h was 26.4 ± 2.3% (±s.e.m., n = 15), and the average cyclic GMP increase in 2 min was 20.8 ± 4.8 times the basal level (±s.e.m., n = 9). Average responses to 10⁻⁴ M MNNG are listed in Table 2. NA, Not applicable.

Increased intracellular cyclic GMP does not correlate with protein discharge from pancreatic acinar cells

THE pancreatic acinar cell discharges secretory proteins in response to cholecystokinin-pancreozymin (CCK-PZ) and its analogues, or to cholinergic agonists such as carbamylcholine (carbachol). The mechanisms for stimulus-secretion coupling in the acinar cell are not known, although intracellular and, to some extent, extracellular Ca²⁺ are required¹⁻⁴, and a transient 10- to 20-fold increase in intracellular levels of guanosine cyclic 3'5'-monophosphate (cyclic GMP) accompanies onset of

induced discharge^{5,6}. It has been proposed⁵ that cyclic GMP may be a direct mediator of hormone-stimulated discharge, with the Ca²⁺-dependent event either causing, or being caused by, increases in cyclic GMP^{7,8}. To test this hypothesis, we have monitored the effects of several substances on secretory protein discharge and intracellular cyclic GMP levels in guinea pig

pancreatic acini. Our results, reported here, are not consistent with a role for cyclic GMP in a direct causative pathway leading to discharge, as four of the compounds tested (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), sodium nitrite, hydroxylamine and sodium nitroprusside) elevated levels of cyclic GMP but did not trigger discharge, although acini treated with these agents remained competent to respond to natural secretagogues. Conversely, the phorbol ester 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) did not affect cyclic GMP levels, but did elicit discharge. In contrast to observations on cyclic GMP, the requirement for extracellular Ca^{2+} correlated well with discharge. Our findings therefore do not support the hypothesis that cyclic GMP is directly involved in stimulus-secretion coupling, but are consistent with such a role for Ca^{2+} . A preliminary report of this work has appeared⁹.

Compounds were chosen because they increased cyclic GMP in other cell types. TPA, a tumour-promoting alkaloid¹⁰, increases cyclic GMP in skin fibroblasts, with concomitant stimulation of DNA synthesis¹¹. TPA also activates platelets and increases platelet cyclic GMP¹². MNNG and hydroxylamine activate guanylate cyclase^{13,14} and elevate intracellular cyclic GMP^{13,15} in liver and other tissues. Similar effects occur with sodium nitrite^{13,16}, sodium nitroprusside¹⁷ and ascorbic and dehydroascorbic acids¹⁸⁻²⁰. These compounds may activate guanylate cyclase by direct oxidation of a reactive group on the enzyme, perhaps by a mechanism involving nitroxide or another free radical as an intermediate²⁰.

MNNG did not trigger discharge in pancreatic acini, but dramatically increased cyclic GMP levels (Table 1). In a representative experiment (Table 1, experiment 1), 10^{-9} M caerulein, a decapeptide analogue of CCK-PZ²¹, triggered the net discharge (total discharge minus unstimulated discharge) of 47% of secretory proteins in 2 h. Caerulein also caused a sixfold increase in cyclic GMP after 2 min. In contrast, MNNG did not stimulate discharge, although 10^{-3} M and 10^{-4} M MNNG caused 45- and 58-fold increases, respectively, in cyclic GMP levels. Acini treated with MNNG are, however, still competent to discharge secretory proteins in response to caerulein (Table 1, experiment 2). The fact that extremely high cyclic GMP levels

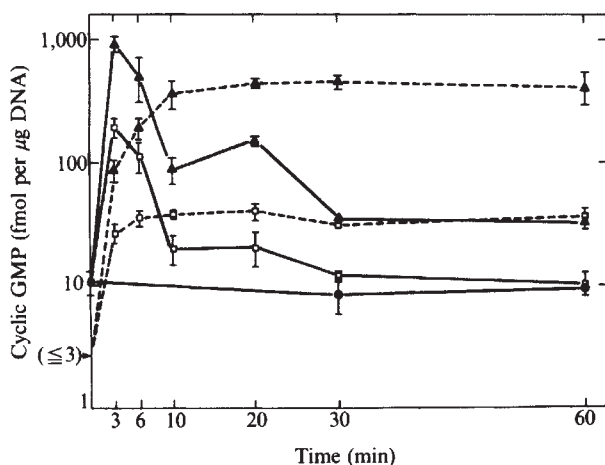


Fig. 1 Effect of MNNG 10^{-4} M and caerulein 10^{-9} M on intracellular and extracellular cyclic GMP levels in pancreatic acini as a function of incubation time. Acini were preincubated for 10 min at 37°C in KRH (see Table 1) and then received either caerulein ($\square$), MNNG ($\blacktriangle$) or no additions ($\bullet$). At the times indicated, the incubation mixtures were centrifuged and the media removed. Acini (—) and media (---) were analysed separately for cyclic GMP (see Table 1). Cyclic GMP levels in cells and media were normalised to DNA content of cell pellets. Note that cyclic GMP levels are plotted on a log scale. After 3 min, more than 95% of the total cyclic GMP is intracellular, justifying the procedure used in experiments shown in Tables 1 and 2 in which acini and media were not separated. Error bars represent ± 1 s.d. for triplicate incubations from the same experiment.

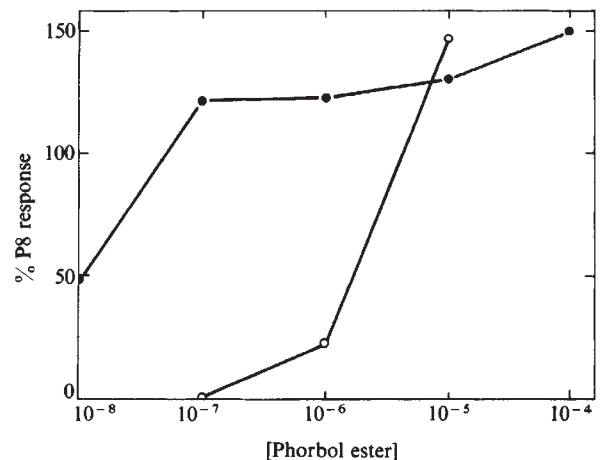


Fig. 2 Effect of TPA ($\bullet$) and 4-*O*-methyl-TPA ($\circ$) on discharge from pancreatic acini. Discharge assays were carried out as described in Table 1. Phorbol esters were added from a 200-fold concentrated stock solution in dimethyl sulphoxide, which, when added alone at a concentration of 0.5%, had no effect on discharge. As experiments with TPA and 4-*O*-methyl-TPA were carried out on different days, the net discharge (total discharge minus resting discharge) was normalised to the net response elicited in the same experiment by the C-terminal octapeptide of CCK-PZ (P8), this being set at 100%. Data points are averages of duplicate incubations from single experiments. The normalised response to 10^{-7} M TPA in 2 h was $90 \pm 17\%$ ($\pm$ s.e.m., $n = 3$). CCK-PZ octapeptide was from Dr Miguel Ondetti (Squibb) TPA and 4-*O*-methyl-TPA were from Consolidated Midland.

did not affect the cellular response to caerulein suggests that cyclic GMP does not inhibit Ca^{2+} entry into pancreatic acini, a mechanism that has been postulated for smooth muscle¹⁷. In addition, stimulation of discharge by 10^{-5} M carbachol was also unaffected by 10^{-4} M MNNG (data not shown). The lack of discharge with MNNG is probably not due to its inactivation, as repeated addition failed to elicit discharge (Table 1, experiment 2). We have also determined that 10^{-4} M MNNG does not stimulate discharge of amylase from acini. Therefore, MNNG does not cause zymogen granule release, as amylase discharge primarily reflects this final secretory step.

The cyclic GMP increase stimulated by MNNG is similar to that triggered by caerulein. Both are partially inhibited by the metabolic inhibitor carbonyl cyanide meta-chlorophenyl hydrazone (CCCP), and neither is affected by removing extracellular Ca^{2+} . The increase in cyclic GMP after 2 min in response to 10^{-9} M caerulein or to 10^{-4} M MNNG was inhibited by $83 \pm 3\%$ and $34 \pm 4\%$, respectively, by 10^{-5} M CCCP (mean $\pm$ s.e.m., $n = 3$). CCCP also inhibits cyclic GMP increases stimulated by carbachol⁵. In contrast, removing extracellular Ca^{2+} (in the presence of 10^{-4} M EGTA) did not affect the increase in cyclic GMP caused by caerulein or MNNG. A Ca^{2+} -independent increase in cyclic GMP has also been observed in response to caerulein^{7,8}, carbachol^{7,8} and the Ca^{2+} ionophore A23187 (ref. 8).

The time dependence for elevation of intracellular cyclic GMP by 10^{-9} M caerulein or 10^{-4} M MNNG is shown in Fig. 1 (solid lines). Within 3 min, caerulein stimulated an 18-fold increase in cyclic GMP, whereas the increase in response to MNNG was 87-fold. This larger increase with MNNG was consistently observed (see Table 1). Intracellular cyclic GMP levels decreased rapidly between 3 and 10 min after addition of the agents, and by 30 min the cyclic GMP concentration in caerulein-treated acini returned to unstimulated levels. The apparently higher level in MNNG-treated acini after 30 or 60 min may be due to cyclic GMP in the extracellular medium trapped within the cell pellets during centrifugation.

Cyclic GMP appears in the medium within 3 min after addition of caerulein or MNNG (Fig. 1, dotted lines) and plateaus

Table 2 Effect of various agents on cyclic GMP level and discharge response

Agent	Final concentration	Ratio of cyclic GMP (stimulated) to cyclic GMP (bkg) (2 min)	Normalised net discharge response (10 ⁻⁹ M caerulein = 100%)	
			Agent alone (%)	Agent plus 10 ⁻⁹ M caerulein (%)
MNNG	10 ⁻⁴ M	79.9 ± 10.1 (12)	2.0 ± 1.6 (14)	112 ± 11 (4)
NaNO ₂	10 ⁻³ M	22.4	-2.9	127
NH ₂ OH	10 ⁻³ M	162.0	-2.0	123
Na nitroprusside	10 ⁻³ M	93.3	4.5	113
Methylnitrosourea	10 ⁻³ M	1.9	0.2	97.6
Dehydroascorbic acid	10 ⁻² M	0.8	0.03 ± 0.05 (3)	96.3
Dehydroascorbic acid	10 ⁻³ M	0.65 ± 0.05 (3)	-5.2 ± 1.2 (3)	81.3
Ascorbic acid	5 × 10 ⁻³ M	0.8	-0.9	92.8

Assays for discharge and cyclic GMP were as in Table 1. Incubation times were 2 min for cyclic GMP measurements and 2 h for discharge assays. The agents listed were added to acini as 100-fold concentrated stock solutions in acetone (MNNG (Aldrich), methylnitrosourea (K + K), dimethyl sulphoxide (DMSO) (for 10⁻³ M dehydroascorbate, K + K), or 0.15 M NaCl (NaNO₂, NH₂OH, sodium nitroprusside (all Fisher), ascorbate (Sigma)). A 1% concentration of acetone or DMSO had no effect on cyclic GMP levels or discharge rate. Dehydroascorbate 10⁻² M was added as a twofold concentrated solution in KRH. Cyclic GMP levels in treated acini were divided by the level in untreated acini (bkg) in each experiment to yield the ratios listed in Table 2. The average cyclic GMP level in untreated acini was 23.0 ± 19.4 fmol per µg DNA (±s.e.m., n = 19). Discharge rates were corrected by subtracting the resting rate and normalised by setting the value for 10⁻⁹ M caerulein at 100%. Negative values indicate rates slightly below the resting rate. Where indicated, values are the mean ±s.e.m. for the number of experiments in parentheses. Otherwise, the data represent the mean of duplicate or triplicate incubations from one experiment.

after 10 min. Cyclic GMP is also released in response to carbachol²². Whatever the mechanism for release of cyclic GMP, in the case of MNNG it cannot be exocytosis of possible zymogen granule-associated cyclic GMP, as MNNG does not cause granule discharge.

The effects of six other compounds known to elevate cyclic GMP levels in other tissues¹¹⁻²⁰ were also tested on pancreatic acini (Table 2). None of these substances elicited discharge, although sodium nitrite, hydroxylamine and sodium nitroprusside produced significant increases in cyclic GMP levels within 2 min. As is the case with MNNG, acini treated with each of the compounds remained responsive to caerulein.

In contrast to MNNG, TPA stimulated optimal discharge at doses from 10⁻⁷ M to 10⁻⁴ M (Fig. 2), but had no effect on cyclic GMP levels. TPA- and hormone-induced discharges were similar: both were inhibited by 10⁻⁵ M CCCP or by 10⁻⁴ M EGTA in Ca²⁺-free medium. In addition, cell morphology after a 2-h incubation in 10⁻⁶ M TPA was consistent with normal stimulation when observed by electron microscopy. Amylase discharge in response to TPA paralleled discharge of labelled secretory proteins. The methylated TPA derivative, 4-O-methyl-TPA, was 100-fold less potent as a secretagogue (Fig. 2) and has greatly reduced tumour-promoting activity¹⁰, suggesting that the mechanisms by which TPA acts as a tumour promoter and a secretagogue may be related. Finally, neither TPA nor 4-O-methyl-TPA significantly altered cyclic GMP levels, regardless of incubation time (10 s–1 h) or concentrations used (10⁻⁷–10⁻⁴ M).

In conclusion, our experiments do not support the hypothesis⁵ of a causal relationship between increased intracellular cyclic GMP and secretory protein discharge in pancreatic acinar cells. MNNG, hydroxylamine, sodium nitrite and sodium nitroprusside all increase cyclic GMP but do not elicit discharge (Table 1, Fig. 1, Table 2). Conversely, TPA triggers discharge (Fig. 2) but does not increase cyclic GMP. These findings are complemented by a recent report that cyclic nucleotide phosphodiesterase inhibitors elevate cyclic GMP but do not elicit discharge in rat pancreas²³. We have also determined that neither MNNG, TPA nor caerulein affect cyclic AMP in guinea pig pancreatic acini. The possibility remains that cyclic GMP pools formed in response to MNNG and caerulein are located within different cellular compartments. This question could be resolved by immunocytochemistry²⁴.

Assuming that cyclic GMP is not a direct mediator of discharge, it seems that the most likely candidate is Ca²⁺. Caerulein, carbachol and TPA failed to elicit discharge when Ca²⁺ was omitted from the medium and 10⁻⁴ M EGTA was included. Other secretagogues, including A23187 (ref. 25), require extracellular Ca²⁺. Recently, Chandler and Williams³ have

suggested that secretagogues cause Ca²⁺ release from an intracellular membrane site which presumably must be replenished by Ca²⁺ influx across the plasma membrane. Whatever the mechanism for Ca²⁺ mobilisation, the question remains as to the nature of the proximal event following the increase in intracellular free Ca²⁺.

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- Williams, J. A. & Chandler, D. *Am. J. Physiol.* **228**, 1729–1732 (1975).
- Kano, T. & Nishimura, O. *J. Physiol., Lond.* **257**, 309–324 (1976).
- Chandler, D. E. & J. A. Williams. *J. Cell Biol.* **76**, 371–385 (1978).
- Williams, J. A. *Cell Tissue Res.* **192**, 277–284 (1978).
- Haymovits, A. & Scheele, G. A. *Proc. natn. Acad. Sci. U.S.A.* **73**, 156–160 (1976).
- Albano, J. Bhoala, K. D. & Harvey, R. F. *Nature* **262**, 404–406 (1976).
- Scheele, G. A. & Haymovits, A. *Ann. N.Y. Acad. Sci.* **307**, 648–652 (1978).
- Christophe, J. P., Frandsen, E. K., Conlon, T. P., Krishna, G. & Gardner, J. D. *J. biol. Chem.* **251**, 4640–4645 (1976).
- Günther, G. R. & Jamieson, J. D. *J. Cell Biol.* **75**, 415a (1977).
- Boutwell, R. K. *Crit. Rev. Tox.* **2**, 419–443 (1974).
- Estensen, R. D. *et al.* in *The Regulation of Proliferation in Animal Cells* (ed. Clarkson, B. & Baserga, R.) 627–634 (Cold Spring Harbor Laboratory, New York, 1974).
- White, J. G., Goldberg, N. D., Estensen, R. D., Haddox, M. K. & Rao, G. H. R. *J. clin. Invest.* **52**, 89a (1973).
- DeRubertis, F. R. & Craven, P. A. *Science* **193**, 897–899 (1976).
- Kimura, H., Mittal, C. K. & Murad, F. *J. biol. Chem.* **250**, 8016–8022 (1975).
- Kimura, H., Mittal, C. K. & Murad, F. *Nature* **257**, 700–702 (1975).
- Katsuki, S. & Murad, F. *Molec. Pharmacol.* **13**, 330–341 (1977).
- Schultz, K.-D., Schultz, K. & Schultz, G. *Nature* **265**, 750–751 (1977).
- Haddox, M. K. *et al.* in *Growth Kinetics and Biochemical Regulation of Normal and Malignant Cells* (eds Drewinko, B. & Humphrey, R. M.) 255–269 (Williams and Wilkins, Baltimore, 1977).
- Goldberg, D. D. *et al.* in *Advances in Cyclic Nucleotide Research* (eds Greengard, P. & Robinson, G. A.) 101–130 (Raven, New York, 1978).
- Haddox, M. K., Stephenson, J. H., Moser, M. E. & Goldberger, N. D. *J. biol. Chem.* **253**, 3143–3152 (1978).
- Ersparmer, V. A. *Rev. Pharmacol.* **11**, 327–350 (1971).
- Kapoor, C. L. & Krishna, G. *Science* **196**, 1003–1005 (1977).
- Heisler, S. & Lambert, M. *Can. J. Physiol. Pharmacol.* **56**, 395–399 (1978).
- Ong, S. H., Whitley, T. H., Stowe, N. W. & Steiner, A. L. *Proc. natn. Acad. Sci. U.S.A.* **72**, 2022–2026 (1975).
- Williams, J. A. & Lee, M. *Biochem. biophys. Res. Commun.* **60**, 542–548 (1974).
- Günther, G. R., Schultz, G. S., Hull, B. E., Alicea, H. A. & Jamieson, J. D. *J. Cell Biol.* **75**, 368a (1977).
- Amsterdam, A., Solomon, T. E. & Jamieson, J. D. in *Meth. Cell Biol.* **20**, 362–378 (1978).
- Matsuzawa, H. & Nirenberg, M. *Proc. natn. Acad. Sci. U.S.A.* **72**, 3472–3476 (1975).
- Steiner, A. L., Parker, C. W. & Kipnis, D. M. *J. biol. Chem.* **247**, 1106–1113 (1972).
- Burton, K. *Biochem. J.* **62**, 315–323 (1956).
- Amsterdam, A. & Jamieson, J. D. *J. Cell Biol.* **63**, 1057–1073 (1974).

Rabbit skeletal myosin isoenzymes from fetal, fast-twitch and slow-twitch muscles

It is generally accepted that myosin in vertebrate fast-twitch and slow-twitch muscles is structurally¹⁻⁴ and immunochemically⁵⁻⁸ distinct and exists in several isoenzymatic forms⁹⁻¹⁴. The nature of myosin in developing mammalian skeletal muscles is controversial. Some authors^{15,16} consider it to be identical to adult fast-twitch myosin on the basis of light-chain composition and histochemistry. Others⁸ suggest that it may be a mixture of fast-twitch and slow-twitch myosins. Still others^{4,17} maintain it is a distinct fetal form of myosin differing in primary structure from adult myosins. In this report we show that (1) myosin in both neonatal and adult fast-twitch muscles of the rabbit exists in three electrophoretically distinct isoenzymatic forms which differ in light-chain composition, and (2) the two-dimensional peptide map of the heavy chains of neonatal muscle myosin is distinct from maps of both fast-twitch and slow-twitch myosins. These results clearly establish the existence of fetal myosin isoenzymes in developing mammalian muscles and resolve apparent contradictions in the literature.

Pyrophosphate gel electrophoresis⁹ has now been established as a sensitive and reliable method of resolving myosin isoenzymes differing in light-chain^{13,18} or heavy-chain¹⁹ composition. On analysis in this system, myosin from slow-twitch soleus muscle of the adult rabbit showed a single component (Fig. 1a). Adult fast-twitch longissimus dorsi muscle (Fig. 1b) showed three components (FM₁, FM₂ and FM₃) which migrated faster than the slow-twitch myosin. The longissimus dorsi muscle from a 2-day-old rabbit (Fig. 1c) also showed three components (fm₁, fm₂ and fm₃) which were electrophoretically faster than fast-twitch myosin components. However, a mixture of fast-twitch and fetal myosins (Fig. 1d) showed that fm₃ co-migrated with

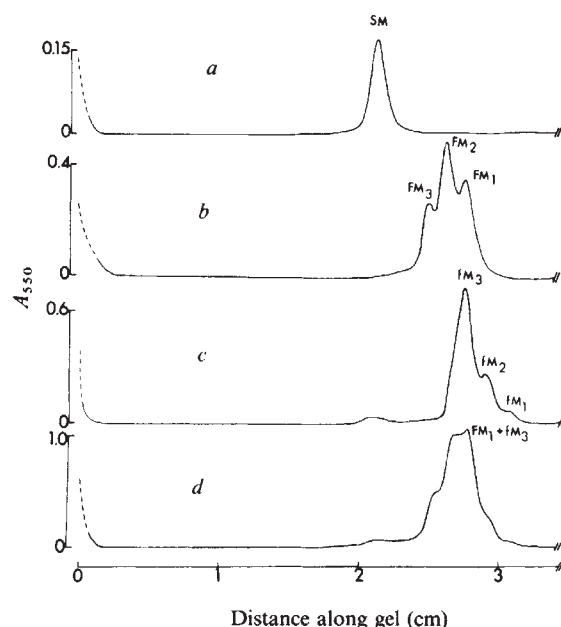


Fig. 1 Pyrophosphate gel electrophoresis of native myosins in polyacrylamide gels. Myosin was extracted and analysed in 4% polyacrylamide gels as described in refs 9 and 13. The curves show densitometer tracings of gels stained with Coomassie Brilliant Blue R. a, Slow-twitch myosin (SM) from adult rabbit soleus muscle; b, fast-twitch myosin components (FM₁, FM₂ and FM₃) from adult rabbit longissimus dorsi muscle; c, fetal myosin components (fm₁, fm₂ and fm₃) from the longissimus dorsi muscle of a 2-day-old rabbit; d, mixture of b and c, showing that FM₁ co-migrates with fm₃.

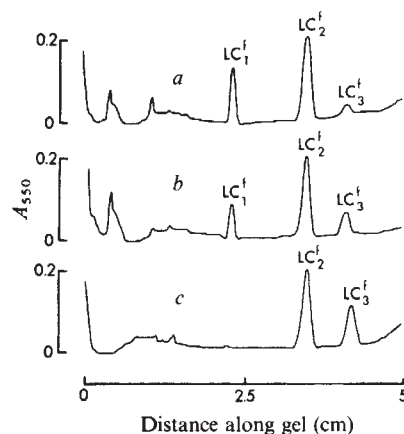


Fig. 2 SDS gel electrophoresis of myosin light chains from electrophoretically purified adult fast-twitch myosin components. After pyrophosphate gel electrophoresis, portions of the gel containing individual components were cut out and analysed for myosin light chains by SDS gel electrophoresis in 12.5% polyacrylamide gel as described in ref. 13. The curves are densitometer tracings showing the distribution of the three fast-twitch myosin light chains from three consecutive slices of the same pyrophosphate gel: a, LC₁^f (or A1), LC₂^f (DTNB light chain) and LC₃^f (A2), in FM₃; b, FM₂; c, FM₁. LC₁^f is absent from FM₁, the ratio LC₂^f:LC₃^f for this component is approximately 1:1. In FM₂, all three light chains are present, the ratio LC₁^f:LC₂^f:LC₃^f was approximately 1:2:1. A small amount of LC₃^f is present in the FM₃ slice from this particular pyrophosphate gel. Its presence is due to a slight contamination with FM₂ in this slice, as it is absent from the FM₃ slice of other gels. Because gels were sliced using a stained companion gel as a guide, it was difficult to avoid some contamination in one of the three consecutive slices. Where FM₃ was not contaminated by FM₂, the ratio LC₁^f:LC₂^f was approximately 1:1. Apart from problems arising from contamination by neighbouring components, the distribution and stoichiometry of the light chains described here have been confirmed in several electrophoretic runs.

FM₁. Essentially the same profile of fetal components as shown in Fig. 1c was obtained from the developing slow-twitch soleus muscle of 2-day-old rabbits.

The isoenzymatic nature of the six components from adult fast-twitch and fetal myosins is shown by the analysis of their light-chain composition by methods described previously¹³. Figure 2 shows the result of light-chain analysis for FM₃ (Fig. 2a), FM₂ (Fig. 2b) and FM₁ (Fig. 2c). These results suggest that the light-chain composition in rabbit fast-twitch myosin isoenzymes is identical to the homologous chick fast-twitch myosin isoenzymes¹³, being (LC₂^f)₂(LC₃^f)₂, LC₁^f(LC₂^f)₂LC₃^f and (LC₁^f)₂(LC₂^f)₂, respectively.

Myosin from developing mammalian muscles contains three light chains which are indistinguishable from LC₁^f, LC₂^f and LC₃^f on SDS gel electrophoresis^{15,20-23}. Analysis of light chains from individual fetal myosin components (Fig. 3) showed that fm₁, fm₂ and fm₃ are homologous to FM₁, FM₂ and FM₃, respectively, in light-chain composition. Although FM₁ co-migrated with fm₃, their different light-chain compositions show them to be different isoenzymes. As light chains constitute only a small fraction of the total molecular mass of native myosin, the difference between fetal and fast-twitch myosins with respect to their electrophoretic behaviour suggests that the heavy chains of these two types of myosin may be structurally different. This is borne out by peptide mapping of myosin heavy chains.

Cyanogen bromide digestion is a convenient method of treating myosin heavy chains for peptide mapping, as the number of peptide fragments is kept to a manageable level compared with the use of trypsin. Two-dimensional mapping of cyanogen bromide peptides has been used successfully to examine structural differences in the heavy chains of ventricular myosin isoenzymes in the rat¹⁹ and is here applied to the analysis of the heavy chains of the three types of skeletal myosin. The maps

(Fig. 4) for fast-twitch (adult longissimus dorsi, 3-day-old rabbit) and slow-twitch (adult soleus) myosins are clearly different. These maps are consistently reproducible from experiment to experiment. The greater complexity of the map for fast-twitch myosin heavy chains when compared with maps of the other two myosins may be related to the known heterogeneity of the fast-twitch myosin heavy chains^{24,25}. Fetal peptides mapped in the more acidic regions of the gel compared with those of the other two myosins. This is consistent with the higher electrophoretic mobility of the fetal myosin isoenzymes in pyrophosphate gels. The maps suggest that the heavy chains of the three types of myosin are structurally distinct. This view is corroborated by earlier findings that (1) the patterns of release of tryptic peptides from these three types of myosins are different⁴, and (2) a fetal myosin peptide, differing in amino acid sequence from its homologue obtained from adult muscle, can be isolated from neonatal rabbit muscle¹⁷.

This work provides direct evidence for the existence of a new class of myosin isoenzymes in developing mammalian skeletal muscles which is distinct from those in adult fast-twitch and slow-twitch muscles. An unexpected structural feature of these fetal myosin isoenzymes is that their light-chain types and distribution are so similar to those of the fast-twitch myosin isoenzymes while heavy chains of these two types of myosins are structurally different. This fact has led to the erroneous conclusion that the myosin in developing mammalian muscle is identical to that of adult fast-twitch myosin^{15,16}. It also explains the immunohistochemical reaction between anti-fast-twitch light-chain antibodies and developing fast-twitch and slow-twitch muscle fibres⁸. The reaction between antisera raised against slow-twitch myosin and developing muscle fibres⁸ may be attributable to common antigenic determinants in fetal and slow-twitch myosins.

This work has helped to define the pattern of changes in myosin gene expression during muscle differentiation in the mammal. Contrary to earlier views^{8,15,16}, it shows that both fast-twitch and slow-twitch muscles synthesise the same fetal myosin isoenzymes early in development. Subsequently, fetal myosin isoenzymes are replaced by the appropriate adult fast-twitch or slow-twitch myosin isoenzymes. During the development of the fast-twitch muscle, this change may principally involve the genes for the heavy chains. Recent work²⁶ has

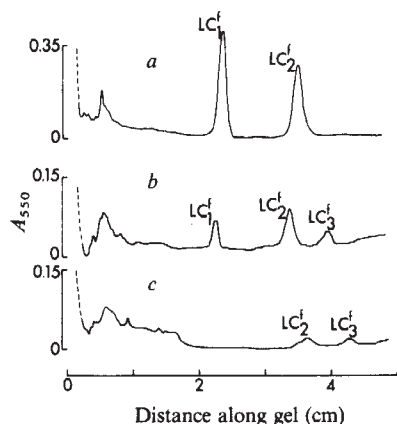


Fig. 3 SDS gel electrophoresis of myosin light chains from electrophoretically purified fetal myosin components. The methods used were the same as those described for Fig. 2. *a*, Light chains from fM₃ showing LC₁^f and LC₂^f only, occurring in approximately equal proportions; *b*, light chains in fM₂, in which LC₁^f:LC₂^f:LC₃^f is approximately 1:2:1; *c*, light chains in fM₁, showing LC₂^f and LC₃^f only and occurring in approximately equal proportions. These results were obtained from consecutive slices of the same pyrophosphate gel. The results for fM₂ and fM₃ have been consistently obtained in many different runs, but fM₁ presents more difficulty as it constitutes only a small fraction of the fetal myosin. However, a similar result has been obtained in another experiment.

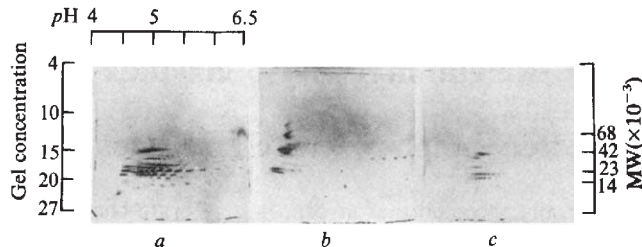


Fig. 4 Two-dimensional cyanogen bromide peptide maps of myosin heavy chains. These maps were obtained for adult fast-twitch myosin (*a*), fetal myosin (*b*) and adult slow-twitch myosin (*c*) using methods described in detail in ref. 19, and briefly outlined below. Myosin heavy chains were separated from carboxymethylated crude myosin extracts through a Sepharose 4B column in a denaturing solvent. The heavy-chain fraction was dialysed and then digested with cyanogen bromide in 70% formic acid at 4 °C for 60 h. The peptides were analysed by isoelectrofocusing in the first dimension between pH 4 and 6.5 and by SDS gel electrophoresis in gradient gels (Gradipore) in the second dimension. Gels were stained in Coomassie Brilliant Blue R. The three slabs were analysed simultaneously in the same electrophoresis unit. Gel concentration along the slab is indicated on the left. The scale on the right marks the positions of molecular weight markers, the numbers giving molecular weights in thousands.

demonstrated that the bulk of the light chains from developing mammalian muscle is indistinguishable in charge as well as in size from those of adult fast-twitch myosin, suggesting that they may be products of the same set of genes. However, in addition, a minor component, called embryonic LC₁, distinct in charge but similar in size to LC₁^f, has also been identified²⁶.

The existence of fetal isoenzymes of myosin in the mammal during the perinatal period is in apparent contrast to the situation in the chick²⁷. Avian fast-twitch and slow-twitch muscles in the 11–14-day chick embryos apparently already synthesise some of the myosin isoenzymes found in the adult muscles. A distinct fetal myosin is apparently absent in both these types of avian muscle at about this time. A possible reason for this difference between rabbit and chick may be that chick fetal myosin is synthesised much earlier than the 14-day embryo. Alternatively, chicken fetal myosin homologous to those demonstrated here may be present in the 11–14-day embryos, but has not been resolved in pyrophosphate gels from adult myosin isoenzymes present during this period of development.

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- Lowey, S. & Risby, D. *Nature* **234**, 81–85 (1971).
- Sarker, S., Sréter, F. A. & Gergely, J. *Proc. natn. Acad. Sci. U.S.A.* **68**, 946–950 (1971).
- Jean, D. H., Guth, L. & Albers, R. W. *Expl. Neurol.* **38**, 458–471 (1973).
- Sréter, F. A., Balint, M. & Gergely, J. *Dev. Biol.* **46**, 317–325 (1975).
- Gröschel-Stewart, U. & Doniach, D. *Immunology* **17**, 981–994 (1969).
- Arndt, I. & Pepe, F. A. *J. Histochem. Cytochem.* **23**, 159–169 (1975).
- Lutz, H., Ermini, M. & Jenny, E. *Histochemistry* **57**, 223–235 (1978).
- Gauthier, G. F., Lowey, S. & Hobbs, A. W. *Nature* **274**, 25–29 (1978).
- Hoh, J. F. Y., McGrath, P. A. & White, R. I. *Biochem. J.* **157**, 87–95 (1976).
- Holt, J. C. & Lowey, S. *Biochemistry* **16**, 4398–4402 (1977).
- Trayer, H. R., Winstanley, M. A. & Trayer, I. P. *FEBS Lett.* **83**, 141–144 (1977).
- Wagner, P. D. *FEBS Lett.* **81**, 81–85 (1977).
- Hoh, J. F. Y. *FEBS Lett.* **90**, 297–300 (1978).
- Weeds, A. *Nature* **274**, 417–418 (1978).
- Pelloni-Müller, G., Ermini, M. & Jenny, E. *FEBS Lett.* **67**, 68–74 (1976).
- Rubinstein, N. A. & Kelly, A. M. *Dev. Biol.* **62**, 473–485 (1978).
- Huszar, G. *Nature new Biol.* **240**, 260–264 (1972).
- Hoh, J. F. Y., McGrath, P. A. & Hale, P. T. *J. molec. cell. Cardiol.* **10**, 1053–1076 (1978).

19. Hoh, J. F. Y., Yeoh, G. P. S., Thomas, M. A. W. & Higginbottom, L. *FEBS Lett.* **97**, 330–334 (1979).
 20. Takahashi, M. & Tonomura, Y. *J. Biochem.* **78**, 1123–1133 (1975).
 21. Jablonski, C. & Kaufman, S. *J. biol. Chem.* **248**, 1056–1062 (1973).
 22. John, H. A. *FEBS Lett.* **39**, 278–282 (1974).
 23. Syrový, I. & Gutmann, E. *Pflügers Arch. ges. Physiol.* **369**, 85–89 (1977).
 24. Starr, R. L. & Offer, G. W. *J. molec. Biol.* **81**, 17–31 (1973).
 25. Pope, B. J., Wagner, P. D. & Weeds, A. G. *J. molec. Biol.* **109**, 470–473 (1977).
 26. Whalen, R. G., Butler-Browne, G. S. & Gros, F. *J. molec. Biol.* **126**, 415–431 (1978).
 27. Hoh, J. F. Y. *FEBS Lett.* **98**, 267–270 (1979).

Fast and slow muscles in tissue culture synthesise only fast myosin

ADULT fast and slow skeletal muscle fibres contain different myosins, a result of innervation by different classes of motoneurons¹; innervation of one fibre type by the opposite motoneurone type leads to a switch in the kind of myosin synthesised^{2,3} within pre-existing fibres^{4,5}. In embryonic muscles, however, the particular myosin isozyme synthesised before and during the early stages of innervation has been a subject of controversy. Some investigators claim that all embryonic muscle fibres—whether destined to be fast or slow in the adult—initially synthesise both fast and slow myosins^{6,7}. Proper innervation would then lead to a selection of one myosin type and repression of the other type within each cell. Others propose that the myosin of embryonic muscles is a distinct myosin, unique to the embryo^{8,9}. On the other hand, we have presented evidence that all fetal muscles have an intrinsic programme which causes them to synthesise fast myosin ini-

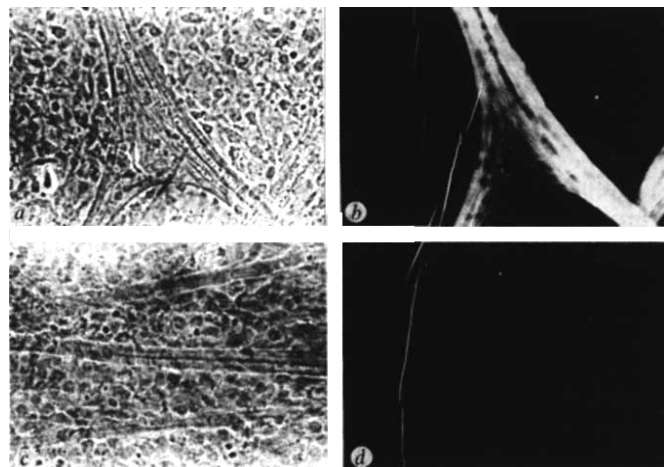


Fig. 1 Staining of cultured pectoralis muscle with fluorescent antimyosins. Muscle was cultured from an 11-d embryonic pectoralis muscle. After 7 d, cultures were fixed in acetone/formaldehyde, stained with affinity-purified, fluorescein-labelled AFHC or ASHC, destained, and photographed on a Zeiss Photomicroscope II equipped with epifluorescence. The production of anti-fast and anti-slow myosin heavy chain IgG has been described previously¹⁵. Affinity-purified AFHC and ASHC were prepared from the IgG by two rounds of absorption. First, each IgG was absorbed to its homologous myosin bound to PAB-cellulose (for example anti-fast IgG absorbed to fast myosin)¹⁵. The absorbed material was recovered and re-absorbed against the heterologous myosin type (for example anti-fast myosin antibody absorbed against slow myosin) to remove any cross-reacting antibodies. The final antibodies are specific to either fast or slow myosin heavy chains and show Ouchterlony reactions identical to those shown previously^{10,15}. *a, c*, Phase micrographs of cultured pectoralis fibres surrounded by mononucleated non-muscle cells. *b*, Same field as (*a*), stained with fluorescent AFHC. *d*, Same field as (*c*), stained with fluorescent ASHC $\times 100$.

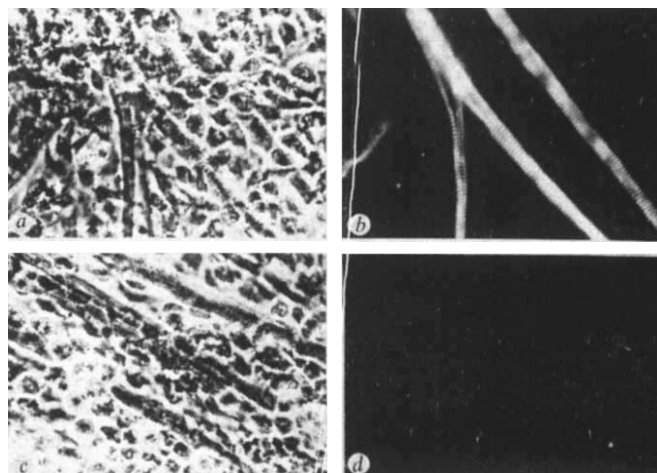


Fig. 2 Staining of cultured ALD muscle with fluorescent antimyosins. Muscle was cultured from a 12-d embryonic ALD muscle. After 7 d, cultures were fixed and stained as described in Fig. 1. *a, c*, Phase micrographs of cultured ALD fibres surrounded by non-muscle cells. (*b*), Same field as (*a*), stained with AFHC. (*d*), Same field as (*c*), stained with ASHC $\times 100$.

ally^{10,11}. After innervation, fast muscles continue synthesising fast myosin, while slow muscles switch to the synthesis of slow myosin. Some studies have corroborated our findings^{12–14}, but in all these studies the earliest fast and slow muscles that could be obtained were already innervated, and the slow muscles already contained a small amount of slow myosin. Accordingly, to examine the initial myosin synthesised in both fast and slow muscles before innervation, we have cultured tissue from chicken muscles destined to be fast or slow muscles in the adult. Muscle fibres in tissue culture are formed in the complete absence of innervation. Whether established from presumptive fast or presumptive slow muscles, all fibres in culture synthesised only fast myosin heavy and light chains.

Cultures of embryonic pectoralis (presumptive fast) and embryonic anterior latissimus dorsi (ALD) (presumptive slow) muscles were stained with affinity purified antibodies specific to adult chicken fast and slow myosin heavy chains. All fibres react only with the anti-fast myosin heavy chain antibody (AFHC) (Figs 1*b*, 2*b*). No reaction can be detected with the anti-slow myosin heavy chain antibody (ASHC) in either the pectoralis (Fig. 1*d*) or ALD (Fig. 2*d*) fibres. Replicating fibroblasts surrounding the fibres contain neither muscle-specific myosin.

These results do not agree with those of several other investigators. Masaki and Yoshizaki⁶ claimed that cultured chicken pectoralis fibres reacted with antibodies against both fast and slow chicken myosins. Their failure to completely purify the myosin used as antigen and failure to affinity purify the antibodies^{6,16} probably led to gross contamination of the anti-myosin antibodies with antibodies to non-myosin proteins, as others have demonstrated¹⁷. Similarly, Gauthier *et al.*⁷ reported that when cross-sections of embryonic rat muscles were stained with antibodies to adult chicken fast and slow myosins, all fibres reacted with both antibodies. We have found, however, that affinity purified anti-chicken myosin antibodies, while staining rat muscle cross-sections, fail to stain the A bands of purified rat myofibrils or show a reaction against rat myosin on immunodiffusion gels (N.A.R. and A. Kelly, unpublished observations). Conversely, Bruggmann and Jenny¹⁸ have shown that anti-rabbit myosin antibodies do not react with chicken myosin.

The ALD and pectoralis cultures might contain small amounts of slow myosin heavy chains not detectable by immunofluorescence. Therefore, cultures were labelled for 24 h with ³⁵S-methionine, washed, and each culture mixed with ~10-fold excess of both unlabelled fast and slow muscle from an

adult chicken. The labelled myosins from the cultures and the unlabelled adult myosins of these mixtures were co-purified and diffused against AFHC and ASHC. As the pectoralis and ALD cultures had been mixed with adult unlabelled fast and slow muscles, each preparation contains unlabelled fast and slow myosin and each shows a reaction against both AFHC and ASHC when the immunodiffusion gels are stained (Fig. 3a, b). An autoradiograph of these gels, however, reveals that only the precipitin line against AFHC (Fig. 3c) contains radioactive myosin—myosin that must have been synthesised in the labelled ALD and pectoralis cultures. Thus, both ALD and pectoralis fibres in culture contain heavy chains reacting identically with AFHC on immunodiffusion plates. The fact that neither culture contains radioactive heavy chains reacting with ASHC (Fig. 3d) cannot be attributed to a failure to reach an equivalence point of antigen and antibody, since the stained gel clearly shows that an equivalence point of ASHC with slow myosin heavy chains was present. The absence of a radioactive precipitin line with ASHC must mean that no slow myosin heavy chains are synthesised in either culture.

Does the radioactive precipitin line in Fig. 3c really represent fast heavy chains or does it merely indicate some cross reaction with an 'embryonic' heavy chain? The radioactive myosin of the precipitin line represents cultured myosin which is less than 10% of the total myosin. This radioactive line exactly overlays the

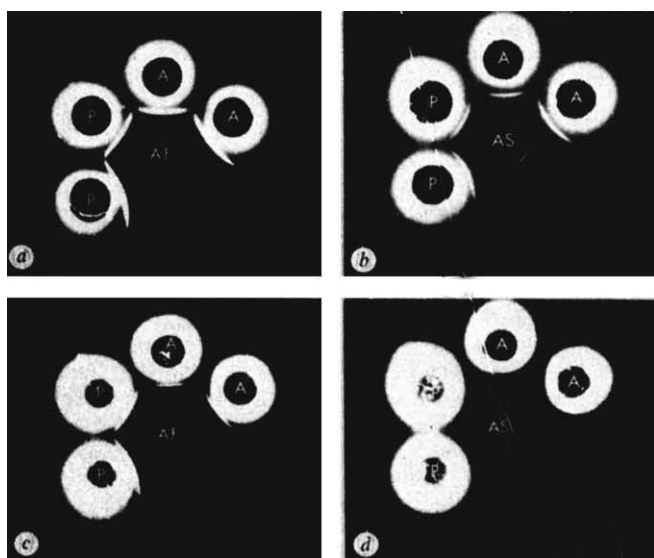


Fig. 3 Myosins from cultured ALD and pectoralis muscles diffused against specific anti-myosins. Myosins were isolated from mixtures of ^{35}S -methionine labelled cultures with unlabelled adult fast and slow chicken muscles. The co-purified myosins were diffused against AFHC and ASHC as previously described¹⁰. After diffusion, the gels were washed, stained with 1% thiazine red, and destained. The gels were then dried and autoradiographed with Royal X-Omat film for 48–72 h at -80° . In (a), AFHC was placed in the centre well. The left two wells (P) contained ^{35}S -methionine labelled myosin from pectoralis cultures mixed with unlabelled adult fast and slow myosins. The right two wells (A) contained ^{35}S -methionine labelled myosin from ALD cultures mixed with unlabelled adult fast and slow myosins. The total myosin concentration in each well was approximately 3 mg ml^{-1} . Since each preparation contained unlabelled adult fast myosin heavy chains, the AFHC reacted with each with a single precipitin line. c, Autoradiograph of (a). Both ALD and pectoralis cultures contained labelled myosin which reacted with AFHC. The precipitin line in (c) exactly coincides with the line in (a). In (b), ASHC was placed in the centre well, while the peripheral wells were filled as in (a). Since each preparation contained unlabelled adult slow myosin heavy chains, the ASHC reacted with each with a single precipitin line. d, Autoradiograph of (b). Neither the ALD nor the pectoralis cultures contained labelled myosin which reacted with ASHC.

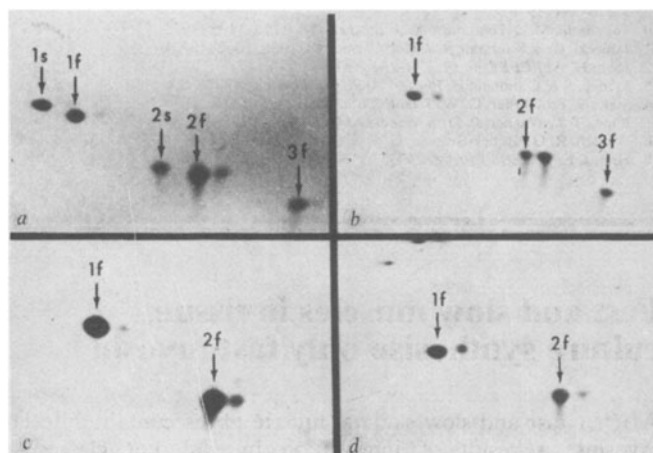


Fig. 4 Two-dimensional electrophoresis of myosin light chains. Myosins isolated by conventional methods¹⁰ were subjected to electrophoresis on isoelectric focusing gels over pH range 4–6, followed by SDS-polyacrylamide gel electrophoresis on 15% acrylamide gels. In each figure, the more acidic region is on the right. a, b, Gels stained with Coomassie brilliant blue. c, d, Fluorographs of labelled cultured myosins which had been mixed with unlabelled adult fast and slow myosins as carriers and markers. Light chains were identified by comparison of fluorographs with the stained gels. Light chains were derived from adult chicken ALD (slow) and pectoralis (fast) myosins (a); adult pectoralis myosin alone (b); ^{35}S -methionine labelled pectoralis culture myosin (c); and ^{35}S -methionine labelled ALD culture myosin (d). Slow myosin light chains are 1s and 2s; fast myosin light chains are 1f, 2f, and 3f. The light chain to the right of 2f is the phosphorylated form of 2f. The chain to the right of 1f may be a secondary modification of 1f; others have claimed that it is a unique embryonic form of the myosin light chain⁸. It is present in both adult and cultured chicken myosins.

precipitin line seen after staining; the stained line, however, represents over 90% unlabelled adult fast heavy chain and less than 10% radioactive heavy chain from the cultures. That the equivalence point of AFHC with adult fast heavy chains and the equivalence point of labelled cultured myosin with 'cross-reacting' antibodies would coincide so exactly is unlikely. When these myosin samples were diluted 1:1 with only unlabelled adult myosin, the radioactive precipitin line still coincided exactly with the stained precipitin line (data not shown). Thus, although we halved the concentration of labelled myosin, its equivalence point was still the equivalence point of adult fast heavy chain with its antibody. Hence, it is unlikely that the radioactive heavy chains from the ALD and pectoralis cultures differ antigenically from adult fast myosin heavy chains.

To examine the myosin light chains more closely, myosins from ^{35}S -methionine labelled ALD and pectoralis cultures (mixed with unlabelled adult fast and slow myosins as carriers and markers) were subjected to electrophoresis on two dimensional gels¹⁹. Figure 4a shows the light chains of a mixture of adult fast and slow light chains. When compared to previous one-dimensional gel analyses¹⁰, which separate proteins only on the basis of molecular weight, some of these light chains show an increase in heterogeneity. Light chain 2f (LC_{2f}) appears as a doublet; the more acidic protein is the phosphorylated form of this light chain. Often, LC_{2s} is also present in its phosphorylated and non-phosphorylated forms, although only the non-phosphorylated chain is evident in Fig. 4.

LC_{1f} also appears as a doublet, with a dominant band accompanied by a minor band at its acidic side. This minor band, which varies in intensity in different preparations, can be seen in all our adult fast and cultured embryonic myosins (see below). Although barely detectable in this mixture of adult fast and slow

myosins (Fig. 4a), it is seen clearly in a different preparation of adult fast myosin alone (Fig. 4b).

When fluorographs are prepared from the cultured ALD (Fig. 4d) and cultured pectoralis (Fig. 4c) muscle myosins after two-dimensional electrophoresis, the dominant light chains co-migrate with adult LC_{1f} and LC_{2f}. In both figures, LC_{2f} is present in both the phosphorylated and non-phosphorylated forms. LC_{1f} is also present as a doublet. Whalen *et al.*⁸ have seen a minor peptide similar to this satellite of LC_{1f} in myosins isolated from fetal, neonatal, and cultured rat fast muscles, although not in myosin from adult rat muscles; for this reason, they consider it an embryonic light chain. However, when RNA from cultured rat myotubes is translated in an *in vitro* system, this minor band is not seen (ref. 20, Fig. 6). In addition, this peptide has been detected in both adult and embryonic muscles of the chicken, cat, and rabbit (A. Kelly and N.A.R. in preparation). Hence, there is no compelling evidence to suggest that this is a distinct embryonic gene product, as proposed by Whalen *et al.*⁸

It has been shown previously that LC_{3f} is not present in cultured chicken muscle or in embryonic chicken muscles *in vivo*^{10,21,22}. The bands migrating near the position of adult LC_{3f} and several others result from fibroblast contamination of the cultures. Neither ALD nor pectoralis cultures show any bands migrating with either LC_{1s} or LC_{2s}.

Muscle fibres in tissue culture, then, whether arising from potential fast or slow muscles *in vivo*, synthesise myosin heavy and light chains identical by several criteria to those in adult fast muscles. Combined with previous *in vivo* studies¹⁰⁻¹⁴, this suggests that all pre-innervated skeletal muscle fibres, regardless of their destiny, synthesise only fast myosin. Thus, the initial expression of the fast myosin gene may be considered an endogenously programmed event in the development of both fast and slow muscles. Some motoneuronal contribution to myogenesis apparently can override this endogenous commitment and cue individual fibres to cease synthesising fast myosin and begin synthesising slow myosin, a process which occurs in normal slow muscle development. This contribution is most likely the specific pattern of impulses generated by the motoneurone²³⁻²⁵.

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1. Buller, A., Eccles, J. & Eccles, R. *J. Physiol. Lond.* **150**, 399-416 (1960).
2. Weeds, A., Trentham, D., Kean, C. & Buller, A. *Nature* **247**, 135-139 (1974).
3. Sreter, F., Elzinga, M., Mabuchi, K., Salmons, S. & Luff, A. *FEBS Lett.* **57**, 107-111 (1975).
4. Pette, D. & Schnez, U. *FEBS Lett.* **83**, 128-130 (1977).
5. Rubinstein, N. *et al. J. Cell Biol.* **79**, 252-261 (1978).
6. Masaki, T. & Yoshizaki, C. *J. Biochem.* **76**, 123-131 (1974).
7. Gauthier, G., Lowey, S. & Hobbs, A. *Nature* **274**, 25-29 (1978).
8. Whalen, R., Butler-Brown, G. & Gros, F. *J. molec. Biol.* **126**, 415-431 (1978).
9. Sreter, F., Balint, M. & Gergely, J. *Dev. Biol.* **46**, 317-325 (1975).
10. Rubinstein, N., Pepe, F. & Holtzer, H. *Proc. natn. Acad. Sci. U.S.A.* **74**, 4524-4527 (1977).
11. Rubinstein, N. & Kelly, A. *Dev. Biol.* **62**, 473-485 (1978).
12. Pelloni-Müller, G., Ermini, M. & Jenny, E. *FEBS Lett.* **67**, 68-74 (1976).
13. Syrový, I. & Gutmann, E. *Pflügers Arch. ges. Physiol.* **369**, 85-89 (1977).
14. Pette, D., Vrbova, G. & Whalen, R. *Pflügers Arch. ges. Physiol.* **378**, 251-257 (1979).
15. Arndt, I. & Pepe, F. *J. Histochem. Cytochem.* **23**, 159-168 (1975).
16. Masaki, T. *J. Biochem.* **76**, 441-449 (1974).
17. Pepe, F. *Cold Spring Har. Symp. quant. Biol.* **37**, 97-108 (1972).
18. Bruggmann, S. & Jenny, E. *Biochim. biophys. Acta* **412**, 39-50 (1975).
19. O'Farrell, P. *J. biol. Chem.* **259**, 4007-4021 (1975).
20. Yablonska, Z. & Yaffe, D. *Differentiation* **8**, 133-143 (1977).
21. Darrow, J. & Stracher, A. *Proc. natn. Acad. Sci. U.S.A.* **68**, 1107-1110 (1971).
22. Chi, J., Rubinstein, N., Strahs, K., & Holtzer, H. *J. Cell Biol.* **67**, 523-537 (1975).
23. Salmons, S. & Vrbova, G. *J. Physiol. Lond.* **201**, 535-549 (1969).
24. Sreter, F., Gergely, J., Salmons, S. & Romanul, F. *Nature* **241**, 17-18 (1973).
25. Salmons, S. & Sreter, F. *Nature* **263**, 30-34 (1976).

Filament geometry and the activation of insect flight muscles

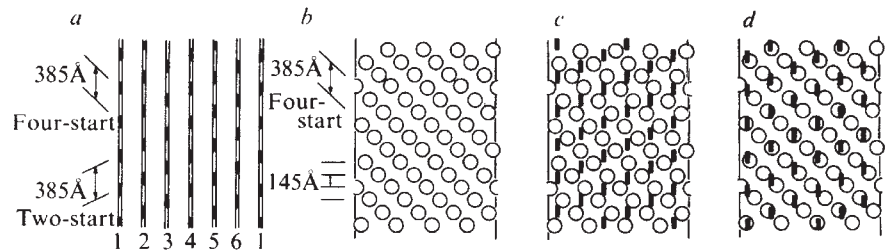
HIGH wingbeat frequencies are required for flight in many insects, and have led to striking specialisations¹. The thorax and wings form a resonant system whose oscillation is sustained by alternating contractions of antagonistic muscles. The action of these flight muscles is to produce only a small-amplitude deformation of the thorax, mechanically amplified into wing movements. The muscles are diverse in arrangement¹ and ultrastructure². In several insect orders, they are of a characteristic 'fibrillar' type whose very regular architecture is well known³. As recognised largely through the work of Pringle⁴, certain properties of fibrillar muscle permit more rapid wingbeats because the oscillation does not depend on pacemaking neural input: the muscles receive only slow and irregular stimulation, and the contraction of each is triggered, after a delay, directly by the stretch applied to it while its antagonist shortens. Studies of isolated glycerol-extracted fibrillar muscle fibres show that unusual properties underlying this sensitivity to stretch are intrinsic to the contractile apparatus itself⁵. ATPase levels and tension are low in calcium-activated fibres at rest length, but are increased by slight extension (0.1-5%). The muscles exhibit both actin-linked and myosin-linked regulation typical of many invertebrate muscles⁶, and the additional regulatory effects of mechanical conditions are not understood. Stretch has been presumed to modify cross-bridge attachment by some unknown effect on the myosin filaments⁴ (possibly transmitted through structures which connect them to the Z-line in certain insect muscles⁷). I suggest here that many aspects of stretch-activation in fibrillar muscles may be explained by the particular geometry of their myosin and actin filaments.

The staggered array of six actin filaments round each myosin filament in the lattice³ is shown in Fig. 1a, 'unrolled' to form a radial projection. The solid bars are regions analogous to 'target areas' of the rigor state³, assumed to be accessible to attachment of active bridges, and they occur every 385 Å along each filament. Two different sets of helical tracks with an axial separation of 385 Å are marked. Figure 1b shows the locations of myosin cross-bridges on the thick filament. These lie on helical tracks whose axial separation is again 385 Å, as indicated by the X-ray pattern of the relaxed muscle⁸; the number of such tracks is not yet firmly established, and Fig. 1b follows the suggestion⁹ that there may be four rather than two⁸ or six¹⁰. Superimposing *a* and *b*, the relationship between cross-bridges and their surrounding envelope of actin targets is seen to depend on the filament alignment. Thus, in *c*, all targets are beyond the 'reach' of the nearby bridges, whereas in *d* all are accessible. Different alignments of actin and myosin filaments are therefore not equivalent: the relationship between tracks of bridges and of targets does not vary throughout each half-sarcomere, but does alter when the alignment is changed by extending the muscle. (When actin and myosin periods are not related, changes occurring locally as a result of extension are largely 'averaged out' over the length of the sarcomere.)

The clear difference between *c* and *d* in Fig. 1 results because *a* and *b* have one set of helical tracks in common, with respect to number, pitch and hand (namely, the four-start tracks of pitch 385 Å), and because targets and bridges are both envisaged as having limited spatial extents. Similar effects result with certain other myosin structures: for example, the two-start myosin helix⁸, if its hand is opposite to that of *b*. (The hand of the actin lattice is known, whereas that of the myosin helix is not³.) In either of these cases, the alternation of match and mismatch repeats after 385 Å of relative movement of the filaments. The six-start myosin helix of Squire¹⁰ permits a much less dramatic difference between match and mismatch, repeating after a movement of only 128 Å.

Filament sliding can therefore greatly influence opportunities for bridge attachment. To illustrate in a general way how these

Fig. 1 Radial projections of actin envelope (a) and myosin surface lattice (b) in insect fibrillar muscle. Bars in a represent regions of thin filaments accessible to bridge attachment. Circles in b represent units of the cross-bridge array, presumably two myosin heads in an as yet unknown relationship. Matching of one set of helical tracks in the two lattices means that average proximity of bridges to attachment regions varies with alignment of the filaments (c and d). How far they can be completely segregated, as in c, depends on their respective dimensions. The size of circles in b reflects a substantial disorder in the cross-bridge array. The matching must have effects on the dynamics of the activated muscle which have no counterpart in cases where a 'vernier' relation exists between thick and thin filament periods. State c is considered the 'ground state' for the muscle at its rest length, whether activated or not. (This state is assumed to be maintained for some structural reason, such as a sterically preferred alignment of the myosin helices with the thin filament components. The preferred alignment will be attainable even in isometric conditions, if a slight dispersion of sarcomere lengths throughout the fibre can occur.) Other states than c will result following stretch, and in activated muscle will generate different levels of activity (see text); during small-amplitude oscillation, the alignment will vary cyclically over some part of the range c-d.



effects can modify conventional muscle dynamics, I assume the four-start matching shown in Fig. 1. The complete mismatch c might explain how rest-length fibrillar muscle fibres can be fully calcium-activated and yet develop little tension (see also Fig. 1, legend). Slight extension of the fibres will increase the accessibility of bridges to targets. The observed response to stretch, in both insect and vertebrate muscles, includes certain rapid transient effects¹¹ followed by delayed tension changes. The latter are generally understood to reflect the equilibration of cross-bridges between different attached and detached states in their new environments, at a rate governed by that of cross-bridge turnover¹². Insect fibrillar muscles are distinctive in that the delayed tension change is large and does not ultimately reverse but instead approaches a different level at the new muscle length. In these terms Fig. 1 suggests a structural reason, hitherto only conjectural, that steady tension might be affected by a length change in fibrillar muscle. It is natural to expect the magnitude of the new tension to depend, perhaps not linearly, on the proximity of bridges to targets. This proximity in turn depends on the amount of extension; it alternately increases and declines with a repeat of 385 Å (about 3% of half-sarcomere length in *Lethocerus*) if Fig. 1 is interpreted literally. But other structural effects might accompany stretch, such as tension-induced changes in myosin helix pitch, non-uniform elongation of sarcomeres, and altered register across the filament lattice. Extension by 3% will then not restore the initial filament alignment immediately, and tension after a stretch will not vary in a periodic manner with the amount of extension. Nevertheless, a periodicity of about 3%, superimposed on other effects, is indeed observed when stretches are applied in sufficiently small and rapid steps¹³, in contrast to earlier observations^{14,15}.

The rapid activation and inactivation of fibrillar muscles could thus be coupled to their small oscillatory length changes through their specialised filament structure. Certain geometrical relationships, accurately preserved throughout the highly regular filament lattice, would ensure that opportunities for bridge attachment can be modulated simultaneously throughout a bundle of muscle fibres in synchrony with the wingbeat cycle. The requisite matching of actin and myosin periods can be related to the unmatched structure in certain non-fibrillar crustacean and insect muscles, through small changes in the 'twist' of the myosin filament⁹. If further work confirms that such geometric effects indeed occur, the present description must be refined in the light of the complex non-linear dynamics of fibrillar muscles. The alternative geometries underlying the sensitivity of activation to stretch should perhaps be manifest also in the rigor state, or when ATP is replaced by its analogues. Finally, it will be interesting to consider how so specialised an adaptation to oscillatory activity can meet the widely varying requirements of flight in different insect species.

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1. Pringle, J. W. S. in *The Physiology of Insecta* Vol. 3, 2nd edn (ed. Rockstein M.) 433-476 (Academic, New York, 1974).
2. Auber, J. *Am. Zool.* **7**, 451-456 (1967).
3. Reedy, M. K. *J. molec. Biol.* **31**, 155-176 (1968).
4. Pringle, J. W. S. *Proc. R. Soc. B* **201**, 107-130 (1978).
5. Jewell, B. R. & Rüegg, J. C. *Proc. R. Soc. B* **164**, 427-459 (1966).
6. Lehman, W., Bullard, B. & Hammond, K. *J. gen. Physiol.* **63**, 553-563 (1974).
7. Auber, J. & Couteaux, R. *J. Microsc.* **2**, 309-324 (1963).
8. Miller, A. & Tregear, R. T. *J. molec. Biol.* **70**, 85-104 (1972).
9. Wray, J. S. *Nature* **277**, 37-40 (1979).
10. Squire, J. M. *J. molec. Biol.* **72**, 125-138 (1972).
11. Huxley, A. F. & Simmons, R. M. *Nature* **233**, 533-538 (1971).
12. Thorson, J. & White, D. C. S. *Biophys. J.* **9**, 360-390 (1969).
13. Abbott, R. H. & Cage, P. E. *J. Physiol., Lond.* **289**, 32P-33P (1979).
14. Chaplain, R. A. *Pflügers Arch. ges. Physiol.* **321**, 223-232 (1969).
15. Pybus, J. & Tregear, R. *J. Physiol., Lond.* **247**, 71-89 (1975).

Transition temperature of excitation-contraction coupling in frog twitch muscle fibres

THE mechanism by which depolarisation of the muscle fibre membrane leads to release of stored Ca^{2+} ions from the sarcoplasmic reticulum, although crucial, is perhaps the least understood stage in excitation-contraction coupling^{1,2}. We report here our investigation of the temperature dependence of this mechanism, using intracellular injection of arsenazo III (refs 3, 4), a Ca^{2+} indicator dye with a fast response time⁵. We have found that the latency between depolarisation of the fibre and the onset of the rise in intracellular free Ca^{2+} is proportional to the reciprocal temperature, but that on an Arrhenius plot this relationship shows a change in slope at a temperature which depends upon the Ca^{2+} concentration in the bathing solution.

Experiments were carried out on the cutaneous pectoris muscle of *Rana temporaria*, stretched to a striation spacing of about 3.6 μm to reduce contraction. The bathing solution contained (in mM): NaCl, 120; KCl, 2; 0, 1.8 or 12 CaCl_2 . In the 0 Ca^{2+} solution, 1 mM EGTA and 5 mM MgCl_2 were added; pH was adjusted to 7.2. The temperature of the solution was controlled by Peltier elements in the base of the chamber, and was measured by a thermistor close to the muscle. Fibres were penetrated with two micropipettes, for voltage recording and dye injection. The dye pipette also served as a current-passing electrode for initiating action potentials, or for voltage clamping. Intracellular Ca^{2+} changes were recorded as previously described^{3,6,7}. Briefly, arsenazo III was injected into a fibre by iontophoresis, and light transmission through the fibre was measured at wavelengths of 532 and 602 nm from a spot of 80 μm diameter. Electrical subtraction of these two signals gave a

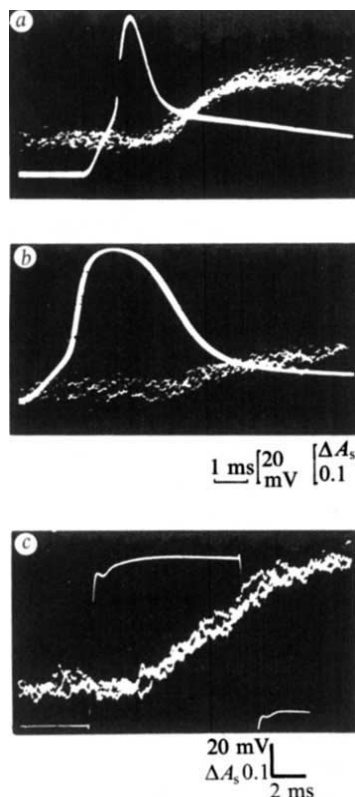


Fig. 1 Delay of the arsenazo III light response following stimulation by action potentials (*a*, *b*), and a voltage-clamped depolarising pulse (*c*). In each record the upper (noisy) trace shows light transmission changes from the arsenazo III-loaded fibre, at the wavelength pair 532–602 nm, and the calibration bars give the standardised change in light absorption, ΔA_s (see ref. 6). The lower traces show the membrane potential. Each record is a superposition of three to four sweeps. The records were obtained from different fibres, at the temperatures indicated. Ringer's solution contained 1.8 mM Ca^{2+} in all cases, and in *c* 2×10^{-6} tetrodotoxin and 30 mM tetraethyl ammonium bromide were also added to the solution. In *a* and *b* the fibres were stimulated by passing depolarising current pulses of 1 ms duration through the dye pipette. In *c* the fibre was voltage clamped, and stimulated by a 10-ms duration depolarising command pulse. Temperature: *a*, 29 °C; *b*, *c*, 7 °C.

record sensitive to Ca^{2+} -dependent changes in absorption of the dye, but in which movement artefacts were reduced.

When a fibre was depolarised to potentials above 0 mV, there was a definite latency between the rise in membrane potential and the onset of the Ca^{2+} response (Fig. 1 and refs 6, 8). This delay increased with decreasing temperature and was similar when the response was elicited by a depolarising pulse after blocking the action potential mechanism with tetrodotoxin, rather than by an action potential. For example, in 12 mM Ca^{2+} Ringer's solution, mean values of the delay following action potentials and depolarising pulses were, respectively, 1.5 ± 0.2 ms (8) and 1.45 ± 0.42 ms (6) at a temperature of 15–16 °C, and 2.6 ± 0.8 ms (7) and 3.0 ± 0.6 ms (6) at 3–4 °C [mean, s.d. (number of fibres)]. Figure 2 shows the temperature dependence of the delay following an action potential, measured in muscle fibres bathed in Ringer's solutions containing various Ca^{2+} concentrations. The latency increased exponentially with the inverse of the absolute temperature (K) in all conditions studied, but at each Ca^{2+} concentration the slope of the relationship changed at a particular temperature. This transition temperature increased with increasing external Ca^{2+} concentration, and was 11 °C in 0 mM Ca^{2+} , 15 °C in 1.8 mM Ca^{2+} and 19.5 °C in 12 mM Ca^{2+} . This effect of Ca^{2+} concentration is probably specific to Ca^{2+} ions and not simply due to a change in divalent cation concentration, as the 0 Ca^{2+} solution contained 5 mM Mg^{2+} .

Another parameter of the excitation–contraction coupling system which displayed a similar temperature dependence to the initial latency, was the time during which a depolarised fibre could maintain an elevated Ca^{2+} level. Figure 3*a* shows a typical light absorbance record from a voltage-clamped fibre which was depolarised from –80 mV to +20 mV for 10 s. An initial large Ca^{2+} response was recorded which declined to the baseline over the duration of the pulse. Figure 3*b* shows an Arrhenius plot of the temperature dependence of the half-duration of this response, measured in a muscle bathed in 0 mM Ca^{2+} Ringer's solution. At temperatures below 11 °C the response half-duration remained constant at about 5.2 s, but at higher temperatures it decreased exponentially with the inverse of the absolute temperature. This transition temperature corresponds closely with that measured for the latency of the arsenazo signal during twitches on 0 mM Ca^{2+} solution. Technical difficulties prevented us from obtaining temperature dependence data at other Ca^{2+} concentrations, but at a temperature of 22.5 °C the response half-duration was slightly dependent on external Ca^{2+} concentration. Mean half-durations were 0 mM Ca^{2+} , 2.8 s (s.e.m. 0.14); 1.8 mM Ca^{2+} , 3.22 s (0.08); 12 mM Ca^{2+} , 3.8 s (0.12). This contrasts with frog slow muscle fibres, in which the duration of the maintained Ca^{2+} response is more strongly dependent on external Ca^{2+} concentration⁹. The peak sizes of the responses were not significantly affected by changes in temperature, suggesting that the shorter duration of the maintained Ca^{2+} response at high temperatures was not the result of exhaustion of Ca^{2+} reserves due to a higher rate of release. This agrees with the view that the amount of Ca^{2+} released during a prolonged depolarisation is controlled by a time-dependent inactivation of the release process^{21,22}.

Possible sources of the latency of the Ca^{2+} response include (1) response time of the dye, and diffusion time of Ca^{2+} within the myoplasm; (2) propagation time of the action potential inwards along the T-system; (3) a delay in the coupling mechanism between T-tubule depolarisation and Ca^{2+} release from the sarcoplasmic reticulum. The response time of arsenazo III is reported as faster than 400 μs at 21 °C (ref. 5) and the complex temperature dependence of the response latency makes it unlikely that the factors in (1) can account for the delay. Active propagation along the T-tubules has a velocity of about 7 cm s^{–1} at 7 °C in frog twitch fibres¹⁰, and would therefore introduce a delay of about 1 ms for conduction to the centre of a

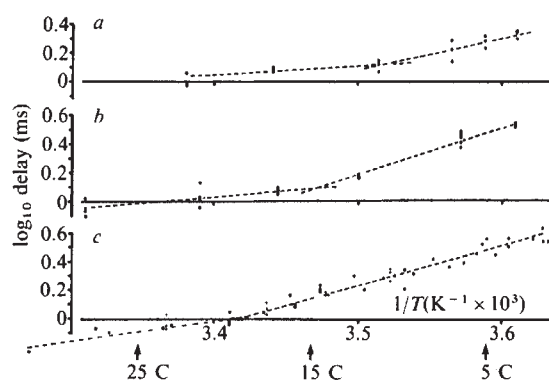


Fig. 2 Arrhenius plots of the log delay of the Ca^{2+} response against T^{-1} . The delay was measured by eliciting action potentials by a 1-ms depolarising pulse applied through the dye pipette, and measuring the latency between the foot of the action potential (about –40 mV) and the onset of the Ca^{2+} response (see Fig. 1). The three graphs show data from muscles bathed in Ringer's solution containing: *a*, 0 mM Ca^{2+} + 1 mM EGTA and 5 mM MgCl_2 ; *b*, 1.8 mM Ca^{2+} ; *c*, 12 mM Ca^{2+} . A different muscle was used for each solution, and results from two muscles (●, and +) are shown in *c*. Each datum point was obtained from a different fibre, and is a mean of nine measurements. Readings were generally obtained from three or four fibres while holding the muscle at a given temperature, and different temperatures were tested in random order. Optical recording time constant was 0.5 ms. The lines drawn through the data were fitted by eye.

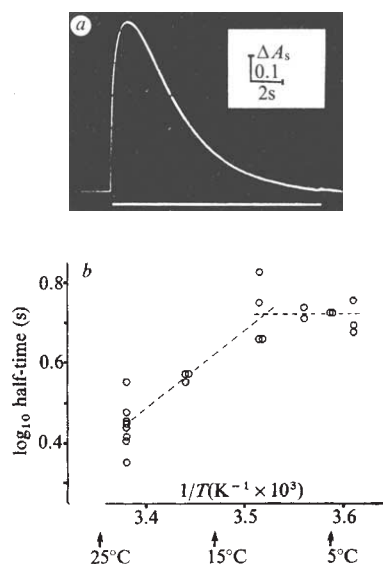


Fig. 3 *a*, Optical record showing the Ca^{2+} -induced change in differential light absorbance at 532–602 nm of an arsenazo III-loaded fibre, in response to a prolonged depolarisation. The fibre was voltage clamped to a potential of -80 mV at rest, and depolarised to $+20$ mV for the duration indicated by the bar. The vertical calibration, ΔA_s , gives the standardised change in light absorbance^{3,6}. Ringer's solution contained 12 mM Ca^{2+} ; temperature 5°C ; recording time constant 33 ms. *b*, Arrhenius plot of the log half-duration of the Ca^{2+} response during prolonged depolarisation. The half-duration was measured as the time for which the light absorbance record exceeded a value of one-half of the peak value. Each point is a single measurement from a different fibre from one muscle. Same experiment as Figs 2*a* and 3*a*.

100- μm fibre. However, the initial rise in the Ca^{2+} response probably results from activation of the sarcoplasmic reticulum at the periphery of the fibre, so that inward conduction time would not be an important factor in determining the minimal delay. A large part of the observed latency may therefore be attributed to the coupling mechanism.

The two component Arrhenius plots shown in Figs 1 and 2 resemble recent results on the elementary properties of drug receptor channels in muscle cells^{11–15}. Possible causes of these temperature transitions include a membrane lipid phase transition^{16–18}, a conformational change in some protein¹⁹ associated with the excitation–contraction coupling process, or different steps of a reaction sequence in the coupling process, being rate limiting in different parts of the temperature range²⁰. Whatever the explanation, the influence of external Ca^{2+} concentration on the transition temperature of the latency in the rise of internal Ca^{2+} during a twitch, suggests that a delay in excitation–contraction coupling is introduced by a structure which is influenced by changes in Ca^{2+} concentration in the bathing medium, and is therefore probably located in the T-tubule membrane. The close similarity of transition temperature, and dependence on extracellular Ca^{2+} , of the duration of maintained Ca^{2+} response further suggests that this is also regulated by a structure in the T-tubule membrane^{21,22}.

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1. Ebashi, S. *A. Rev. Physiol.* **38**, 293–313 (1976).
2. Endo, M. *Physiol. Rev.* **57**, 71–108 (1977).
3. Parker, I. in *Detection and Measurement of Free Calcium Ions in Cells* (eds Ashley C. C. & Campbell A. K.) (Elsevier, Amsterdam, in the press).
4. Scarpa, A., Tiffert, T. & Brinley, F. J. in *Biochemistry of Membrane Transport* (eds Semenza G. & Carafoli E.) 552–566 (Springer, Berlin, 1977).
5. Brown, J. E. *et al. Biophys. J.* **15**, 1155–1160 (1975).
6. Miledi, R., Parker, I. & Schalow, G. *Proc. R. Soc. B* **198**, 201–210 (1977).
7. Miledi, R., Parker, I. & Schalow, G. *J. Physiol., Lond.* **269**, 11–13 (1977).
8. Suarez Kurtz, G. & Parker, I. *Nature* **270**, 746–748 (1977).
9. Miledi, R., Parker, I. & Schalow, G. *Nature* **268**, 750–752 (1977).
10. Gonzalez Serratos, H. *J. Physiol., Lond.* **212**, 777–799 (1971).
11. Anderson, C. R., Cull-Candy, S. G. & Miledi, R. *Nature* **268**, 663–665 (1977).
12. Dreyer, F., Muller, K. D., Peper, K. & Sterz, R. *Pflügers Arch. Ges. Physiol.* **367**, 115–122 (1976).
13. Fischback, G. D. & Lass, Y. *J. Physiol., Lond.* **280**, 527–536 (1978).
14. Feltz, A., Large, W. A. & Trautmann, A. *J. Physiol., Lond.* **269**, 109–130 (1977).
15. Anderson, C. R., Cull-Candy, S. G. & Miledi, R. *J. Physiol., Lond.* **282**, 219–242 (1978).
16. Chapman, D. *Q. Rev. Biophys.* **8**, 185–235 (1975).
17. Wilson, G. & Fox, C. F. *J. molec. Biol.* **55**, 49–60 (1971).
18. Overath, P. & Trauble, M. *Biochemistry* **12**, 2625–2627 (1973).
19. Levy, H. M., Sharon, N. & Koshland, D. E. *Proc. natn. Acad. Sci. U.S.A.* **45**, 785–791 (1959).
20. Dixon, M. & Webb, E. C. *Enzymes* 2nd edn, 758 (Longmans, London, 1964).
21. Caputo, C. *J. Physiol., Lond.* **255**, 191–207 (1976).
22. Caputo, C. *J. Physiol., Lond.* **223**, 483–505 (1972).

Inhomogeneous distribution of filipin–sterol complexes in smooth muscle cell plasma membrane

THE plasma membrane of the smooth muscle cell presents characteristic rows of micro-invaginations or caveolae arranged parallel to the longitudinal axis of the cell^{1–4}. Previous studies have demonstrated that the plasma membrane in the rows contains a much higher density of intramembranous particles than the intervening membrane zones^{2,4}. The polyene antibiotic, filipin, by specifically interacting with cholesterol^{5–7}, produces distinctive alterations in freeze-fractured membranes^{8–11}, and the addition of filipin to aldehyde fixatives has been recently introduced as a cytochemical technique for the freeze-fracture localisation of cholesterol in cell membranes^{12,13}. By applying this technique to smooth muscle cells, we have now obtained morphological evidence that the invaginated, particle-rich bands of the plasma membrane react with filipin to a far greater extent than does the non-invaginated membrane. The two membrane zones may thus differ in cholesterol content.

Strips of smooth muscle from rat large intestine were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 1 h, and incubated overnight at room temperature in the same fixative containing 300 μM filipin (Upjohn) dissolved in a drop of dimethyl sulphoxide (DMSO; final concentration 1%). Glutaraldehyde fixation is aimed at preserving cell ultrastructure by preventing the lysis resulting from filipin-induced permeability

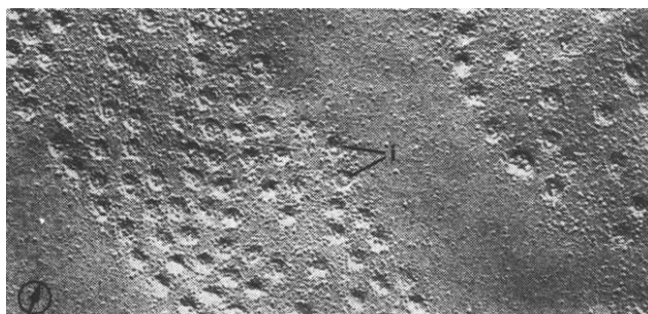


Fig. 1 Freeze-fracture replica of a smooth muscle cell fixed in glutaraldehyde alone. The plasma membrane of the cell has been split to reveal the protoplasmic leaflet or P-face¹⁴. The latter presents two parallel rows of crater-like circular depressions (i) representing the fractured necks of microinvaginations or caveolae. The density of intramembranous particles is higher within the rows than in the intervening membrane. The encircled arrows indicate the direction of platinum-carbon evaporation. $\times 47,000$.

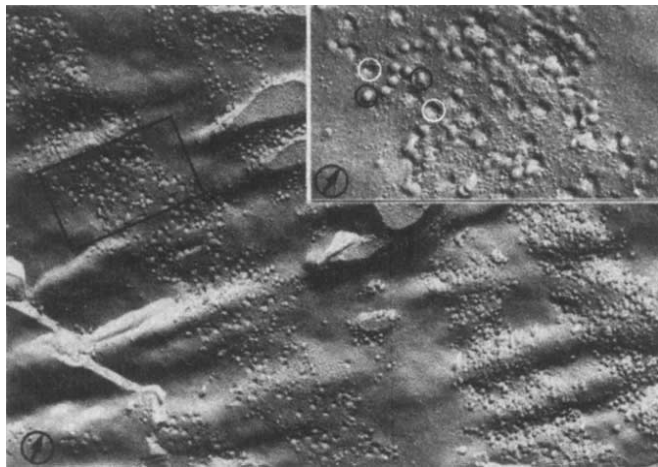


Fig. 2 Freeze-fracture replica of a smooth muscle cell fixed in glutaraldehyde-filipin mixture. The detail of a typical band of invaginations (black rectangle) is shown at high magnification in the inset. Numerous filipin-sterol complexes (black circles) are interspersed among the microinvaginations (white circles), whereas they are virtually absent outside the invaginated membrane regions. Filipin-sterol complexes appear as 25–30 nm protuberances which can be distinguished from the contiguous microinvaginations by the opposite polarity of platinum deposition (the direction of the shadowing is indicated by the encircled arrow). $\times 22,000$. Inset: $\times 44,000$.

changes in living cells⁵. Control muscle was fixed either in glutaraldehyde overnight, or in glutaraldehyde for 1 h and then in glutaraldehyde plus DMSO overnight. After rinsing in 0.1 M cacodylate buffer, pH 7.4, the tissues were cryoprotected with 30% glycerol in the same buffer, mounted on gold disks, and quickly frozen in Freon 22 cooled with liquid nitrogen. Fracturing and shadowing were carried out at -100°C in Balzers BAF 300 and BAF 301 units. Replicas were cleaned by digestion in sodium hypochlorite, washed in distilled water, and recovered on coated 150-mesh copper grids. Freeze-fracture replicas were examined using a Philips EM 300 electron microscope.

Figure 1 illustrates the characteristic freeze-fracture appearance of the smooth muscle cell plasma membrane after aldehyde fixation alone^{1–4}. The protoplasmic or P fracture face¹⁴ shows many crater-like depressions, each one representing the fractured neck of an invagination. These craters are disposed in an orderly manner in parallel bands separated by regions of non-invaginated membrane. As has been previously observed^{2,4}, intramembranous particles are more numerous in the rows of invaginations. Fixation in glutaraldehyde plus 1% DMSO produces freeze-fracture images similar to those obtained with glutaraldehyde alone. When the smooth muscle is in a filipin-containing glutaraldehyde solution, filipin-sterol complexes become recognisable as 25–30 nm protuberances^{8–13} on the fractured plasma membranes (Fig. 2, inset). Filipin-sterol complexes are abundant in the particle-rich regions of the membrane, in which they are interspersed among the invaginations. By contrast, such complexes are virtually absent in the intercalated, particle-poor bands (Fig. 2). Due to the poor penetration of filipin within compact tissues, filipin-sterol complexes were usually confined to the most peripheral portions of the fractured specimens. Nevertheless, whenever found on a smooth muscle cell plasma membrane, they occurred with the selective distribution outlined above.

As filipin specifically binds to cholesterol and other related 3- β -hydroxysterols in cell membranes^{6,7,15}, our observation suggests heterogeneous distribution of these lipids in the smooth muscle cell plasma membrane. The functional significance of this heterogeneity is not clear, but it is worth noting that in the cells studied a higher sterol content is associated with both a higher density of intramembranous particles and morphological specialisation (invaginations) of the plasma membrane. It should

be emphasised here that filipin labelling only represents qualitative evidence for sterol topographical inhomogeneity, and does not permit evaluation of the absolute sterol content of a discrete membrane region. In particular, the virtual lack of filipin reactivity in non-invaginated bands (which are the sites of insertion for actin myofilaments¹⁶) should not necessarily mean that there is no cholesterol there. In these membrane areas, the cholesterol level might be too low to induce the formation of detectable complexes to the same extent as found in invaginated regions.

The presence of a marked heterogeneity in sterol content between contiguous bands of membrane suggests that some restraint must exist in the lateral diffusion of cholesterol in the plane of the native membrane.

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- Devine, C. E., Simpson, F. O. & Bertaud, W. S. *J. Cell Sci.* **8**, 427–443 (1971).
- Wells, G. S. & Wolowyk, M. W. *J. Physiol., Lond.* **218**, 11P–13P (1971).
- Müggli, R. & Baumgartner, H. R. *Experientia* **28**, 1212–1214 (1972).
- Orci, L. & Perrelet, A. *Science* **181**, 868–869 (1973).
- Kinsky, S. C. A. *Rev. Pharmac.* **10**, 119–142 (1970).
- Norman, A. W., Demel, R. A., De Kruijff, B. & Van Deenen, L. L. M. *J. biol. Chem.* **247**, 1918–1929 (1972).
- De Kruijff, B., Gerritsen, W. J., Oerlemans, A., Demel, R. A. & Van Deenen, L. L. M. *Biochim. biophys. Acta* **339**, 30–43 (1974).
- Verkleij, A. J. et al. *Biochim. biophys. Acta* **291**, 577–581 (1973).
- Tillack, T. W. & Kinsky, S. C. *Biochim. biophys. Acta* **323**, 43–54 (1973).
- Kitajima, Y., Takashi, S. & Nozawa, Y. *Biochim. Biophys. Acta* **445**, 452–465 (1976).
- Andrews, L. D. & Cohen, A. I. *J. Cell Biol.* **81**, 215–228 (1979).
- Elias, P. M., Friend, D. S. & Goerke, J. J. *Cell Biol.* **79**, 232a (1978).
- Friend, D. S. & Elias, P. M. *J. Cell Biol.* **79**, 216a (1978).
- Branton, D. et al. *Science* **190**, 54–56 (1975).
- Norman, A. W., Spielvogel, A. M. & Wong, R. G. *Adv. Lipid Res.* **14**, 127–170 (1976).
- Burnstock, G. in *Smooth Muscle* (ed. Bülbirg, E., Brading, A., Jones, A. & Tomita, T.) 1–69 (Arnold, London, 1970).

Visualisation of ¹¹C-flunitrazepam displacement in the brain of the live baboon

THE presence of specific benzodiazepine receptors in the brain of rat^{1,2}, baboon³ and man^{4,5} has been demonstrated *in vitro*. These receptors have also been observed *in vivo* for diazepam⁶ and flunitrazepam⁷. However in such experiments, although the radioactive ligand is injected in the living animal the demonstration of the presence of specific receptors requires brain tissue sampling for quantitative measurement *in vitro*. The fact that carbon-11 is a positron emitter with a half life of 20 min which can be used for very high specific activity labelling of drugs, raises the possibility of external detection of radioactivity in a living subject by positron emission tomography^{8,9}. Using this new methodology the present study demonstrates the displacement of ¹¹C-flunitrazepam by lorazepam in the brain of live baboons.

Eight experiments were carried out with two female baboons (*Papio papio*) weighing 11 and 12 kg. After anaesthetisation with ketamine (15 mg per kg) the animals were intubated, ventilated and curarised by injection of nortoxiferin chloride (0.2 mg per kg as a starting dose followed by 0.1 mg per kg injections when necessary). At the end of the experiments the animals were decurarised using prostigmine. The interval between two experiments on the same animal was a minimum of 2 weeks. The animals were placed supine in a painless position under the positron camera (ECAT-ORTEC), the head inside a

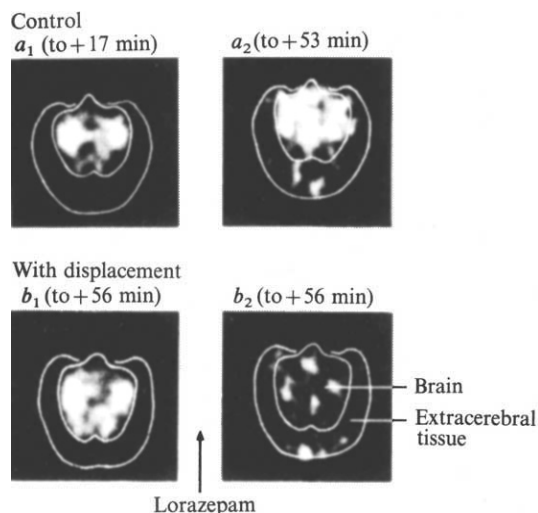


Fig. 1 Brain distribution at different times after i.v. injection of ^{11}C -flunitrazepam observed by positron emission tomography. Comparison of b_2 obtained 26 min after administration of a load of lorazepam ($1.3 \mu\text{M}$ per kg) with a_2 (control experiment) reveals a displacement of the radioactive ligand. See Fig. 2 and Table 1 for quantitative data.

hammock in such a way that a 2-cm thick slice, parallel to and 2 cm above theinion orbital line, was observed by the camera. Flunitrazepam was labelled by ^{11}C -methyl iodide methylation of the nor derivative at a specific radioactivity of $300\text{--}900 \text{ Ci mmol}^{-1}$ at the injection time¹⁰. The labelled drug ($20\text{--}25 \text{ mCi}$), corresponding to $2\text{--}6 \text{ nM}$ per kg weight, was injected intravenously (i.v.) at least 5 h after the ketamine anaesthesia, and the head scans were repeated over 1 h. Arterial blood and expired air samples were taken at given times to measure the radioactivity. In four experiments, lorazepam ($1.3 \mu\text{M}$ per kg) was injected i.v. 30 min after ^{11}C -flunitrazepam to displace the radioactive tracer from the specific receptors. This benzodiazepine was used because of its high capacity to displace flunitrazepam from its specific binding sites⁴. The scans were visualised on a TV monitor and two regions of interest corresponding to cerebral and extracerebral tissues were selected. Quantitative data were obtained by comparing the 'in vivo' activity with that of a control containing a known amount of radioactivity. Results in brain extracerebral tissues and blood were expressed as a percentage of the injected dose per g of tissue. Expired $^{11}\text{CO}_2$ activity was expressed as percentage injected dose. Blood pressure was recorded in some experiments by catheterisation of the femoral artery.

Figure 1 gives the pictures corresponding to one control (a) and one displacement experiment (b). Pictures a_1 and b_1 were

obtained 17 and 20 min after ^{11}C -flunitrazepam injection. They show a high uptake of the labelled drug in the brain as compared with the extracerebral tissue, which cannot be seen on these pictures. On the other hand, 53 min after the tracer injection in the control experiment (a_2) the brain activity is still high but has decreased more rapidly than the extracerebral radioactivity, which is now visible. More important is the difference seen in b_2 taken at 56 min, 26 min after lorazepam injection. The contrast between brain and extracerebral tissue is less in this picture than in a_2 . Figure 2 illustrates the kinetics of ^{11}C -flunitrazepam with and without displacement. In the control experiment the brain radioactivity curve (corrected for physical decay of ^{11}C) shows a sharp decrease during the first 20 min, followed by a more slowly decreasing phase during the next 30 min. In the displacement study lorazepam was injected at the beginning of the low-slope phase of the activity curve and led to a faster brain activity decrease than in the control. By contrast, extracerebral activity remained nearly constant throughout the experiment. Blood activity increased slightly after lorazepam injection.

Table 1 gives the data obtained in four control and four displacement studies. For each experiment the specific radioactivity (when measured) of the ligand at the time of injection, the cerebral, extracerebral, blood and expired $^{11}\text{CO}_2$ activities at the end of the experiment (50–60 min) are indicated. In all tests the extracerebral and blood activities are of the same order of magnitude, unlike that of the brain which is significantly lower after displacement. The presence of $^{11}\text{CO}_2$ in expired air indicates a demethylation of the radioactive ligand.

Modifications in the brain kinetics of high specific activity ^{11}C -flunitrazepam were definitely observed when a load of lorazepam was injected. The amount of radioactive ligand administered ($2\text{--}6 \text{ nM}$ per kg) is of the same order of magnitude as that used by other authors for displacement studies 'in vivo' (ref. 7). Because of the low efficiency of radioactivity detection by external counting on the live animal, the specific radioactivity of the ligand had to be much higher than is generally used for 'in vitro' measurements with tritiated molecules. Although theoretically a specific activity of $10^4 \text{ Ci } \mu\text{mol}^{-1}$ can be reached when labelling with carbon-11, the best value obtained so far did not exceed $0.8\text{--}1.0 \text{ Ci } \mu\text{mol}^{-1}$ at the time of injection¹⁰. A variation in displacement for different anatomical areas could not be observed because of the resolution (12 mm) of the positron camera. Furthermore, the thickness of the slice in the field of view of the detector is 20 mm. In these technical conditions it is difficult in a baboon's brain to differentiate the cortex, in which the density of receptors is high, from the other structures where the concentration is lower.

Chang *et al.*⁷ have shown that after i.v. injection of ^3H -flunitrazepam only that fraction of the ligand bound to membranes is likely to be displaceable. Although bound and free fractions can be separated *in vitro* this is not the case *in vivo*

Table 1 Results of control and displacement studies of ^{11}C -flunitrazepam

	Specific activity (Ci mmol^{-1})	Brain uptake (% per g $\times 10^4$)	Extracerebral uptake (% per g $\times 10^4$)	Brain to extracerebral uptake ratio	Blood (% per g $\times 10^4$)	Expired $^{11}\text{CO}_2$ (% in first hour)
Controls						
1	—	75	58	1.29	—	—
2	650	75	47	1.60	—	—
3	300	100	55	1.80	60	3.2
4	880	120	60	2.0	80	13
Mean $\pm$ s.d.	610	93 ± 22	55 ± 6	1.67 ± 0.3	—	—
After displacement						
1	500	45	45	1.0	50	—
2	—	60	50	1.20	50	10.8
3	570	70	60	1.15	70	13.6
4	620	70	65	1.10	60	9.6
Mean $\pm$ s.d.	563	61 ± 12	55 ± 9	1.11 ± 0.09	58 ± 9.6	—

Specific activity is given at the time of ^{11}C -flunitrazepam injection. Values are given as a percentage of the dose 50–60 min after injection of the radioactive ligand ($20\text{--}25 \text{ mCi}$). In displacement studies lorazepam ($1.3 \mu\text{M}$ per kg) was injected 30 min after ^{11}C -flunitrazepam. The average brain to extracerebral tissue uptake ratio was significantly lower (at the 2% level) after lorazepam injection and shows an apparent 35% displacement.

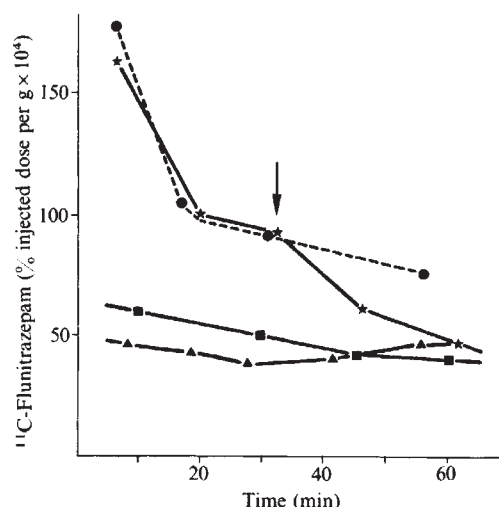


Fig. 2 ^{11}C -Flunitrazepam kinetics. ●, Cerebral uptake in a control experiment. ★, Cerebral uptake in a displacement experiment. Lorazepam ($1.3\ \mu\text{M}$ per kg) was injected (arrow) 30 min after ^{11}C -flunitrazepam. ■, Extracerebral uptake of the displacement experiment. The region measured is shown in Fig. 1. ▲, Blood in the displacement study.

and only the overall activity is measurable, giving an apparent displacement lower than observable when measured *in vitro*. Other physiological parameters, such as blood pressure and blood flow change after administration of a therapeutic dose of lorazepam, redistribution of the tracer in other organs which might modify its availability for brain tissue and the metabolism of the labelled drug itself with loss of the tracer by demethylation, might affect the amplitude of the displacement. In our experiments no modification of arterial blood pressure was observed. A redistribution of the radioactive ligand after displacement can be suspected as a slight increase in blood radioactivity following lorazepam injection was observed in all cases. However, this increase did not modify the extracerebral tissue uptake. Demethylation of the tracer was a significant feature, being variable from one experiment to another. As the *N*-demethylated product, which is not detected, has a pharmacological activity comparable with that of the parent compound, its possible binding to the same specific receptors will contribute to an underestimation of the observed displacement. Because of these points, an accurate quantification of the effect of a pharmacological dose of drug on the displacement of a radioactive ligand is more difficult *in vivo* than *in vitro*. However the data observed in this study correlate well with *in vitro* or semi-*in vivo* experiments. An apparent 35% displacement of ^{11}C -flunitrazepam by $1.3\ \mu\text{M}$ per kg lorazepam agrees well with the ID_{50} values of Chang and Snyder⁷ for clonazepam, flunitrazepam and diazepam in mice. The rapidity with which the displacement is observed, as shown in Fig. 2, corresponds well to the rapid anti-epileptic activity of the benzodiazepines.

These first results indicate that benzodiazepine brain receptors are detected in the live baboon in a non-traumatic way and that the methodology described may be applied to human beings for whom the detector used is better adapted than for animals. The development of new labelling methods with the short-lived radioisotope carbon-11 might also permit the detection in the living brain of other specific receptors and the evaluation of their properties as a function of pharmacological and physiopathological changes.

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1. Squires, R. F. & Braestrup, C. *Nature* **266**, 732–734 (1977).
2. Okada, T. & Mohler, H. *Science* **198**, 849 (1977).
3. Squires, R. F., Naquet, R., Riche, D. & Braestrup, C. *Epilepsia* **20**, 215–221 (1979).
4. Braestrup, C., Albrechtsen, P. & Squires, R. F. *Nature* **269**, 702–704 (1977).
5. Mohler, H., Okada, T., Heitz, Ph. & Ulrich, J. *Life Sci.* **22**, 985 (1978).
6. Williamson, J. M., Paul, S. M. & Skolnick, P. *Nature* **275**, 551–553 (1978).
7. Chang, R. & Snyder, S. *Eur. J. Pharmacol.* **48**, 213–218 (1978).
8. Phelps, M. E. *Seminars in Nucl. Med.* **7**, 337–365 (1977).
9. Soussaline, F. *et al. Eur. J. Nucl. Med.* (in the press).
10. Maziere, M., Godot, J. M., Berger, G. & Comar, D. *J. Radioanal. Chem.* (in the press).

Partial agonists for brain GABA/benzodiazepine receptor complex

BENZODIAZEPINE receptors in the brain¹ are activated by γ -aminobutyric acid (GABA) and by the GABA agonist muscimol *in vitro*². This activation may be related to the GABA-potentiating effects of benzodiazepines observed in electrophysiological studies (see ref. 3 for review). The enhancement of specific ^3H -diazepam binding by GABA agonists is inhibited by bicuculline and thus offers a unique high-affinity binding system for investigations *in vitro* of agonist-antagonist interactions at GABA receptors in the central nervous system. We report here that two GABA-mimetic compounds, 3-aminopropane-sulphonic acid (APS) and isoguvacine, are partial agonists (mixed agonists/antagonists) with intermediate efficacies. Imidazoleacetic acid (IAA) is also a partial agonist but with only marginal agonist activity, and the new GABA-mimetics piperidine-4-sulphonic acid (PSA) and 4,5,6,7-tetrahydroisoxazolo-(5,4-*c*)pyridin-3-ol (THIP) are competitive antagonists to the GABA/benzodiazepine receptor complex.

One group of GABA agonists increases ^3H -diazepam binding to rat brain membranes, prepared as described in Fig. 1 legend. Muscimol ($\text{EC}_{50} = 0.6\ \mu\text{M}$, see Fig. 2 legend), dihydromuscimol ($1.9\ \mu\text{M}$), GABA ($3.8\ \mu\text{M}$), thiomuscimol ($4.2\ \mu\text{M}$), trans-aminocrotonic acid ($7.4\ \mu\text{M}$), (*S*)(-)-5-methylmuscimol ($230\ \mu\text{M}$), (*R*)(+)-4-methyl-GABA ($410\ \mu\text{M}$), (*S*)(-)-4-methyl-GABA ($550\ \mu\text{M}$), and (*R*)(+)-5-methylmuscimol ($2,300\ \mu\text{M}$) increased specific ^3H -diazepam binding to ~200% of the control level⁴ (because of limited supply of the latter three compounds the dose-response curves were stopped before maximal effect was achieved). The EC_{50} values for the nine agonists above as well as for 3-hydroxy-GABA and 3-guanidinopropionic acid⁵ are closely correlated with inhibitory activity on ^3H -GABA binding sites. The EC_{50} values for benzodiazepine receptor activation, however, were 179 ± 32 -fold higher than the respective IC_{50} values for ^3H -GABA binding^{4,6,7} (mean $\pm$ s.e.m. for nine GABA agonists).

Thus three lines of evidence indicate that GABA receptors are involved in the increase in ^3H -diazepam binding: (1) The GABA antagonist bicuculline selectively and completely abolishes the activation induced by muscimol (Fig. 1a and refs 2, 5, 8, 9) and by the eight other agonists described above (data not shown). Bicuculline alone has no effect on this membrane system. (2) The activation of ^3H -diazepam binding is stereoselective for GABA agonists (data above) and highly correlated to the affinity of these same agonists for GABA receptors.

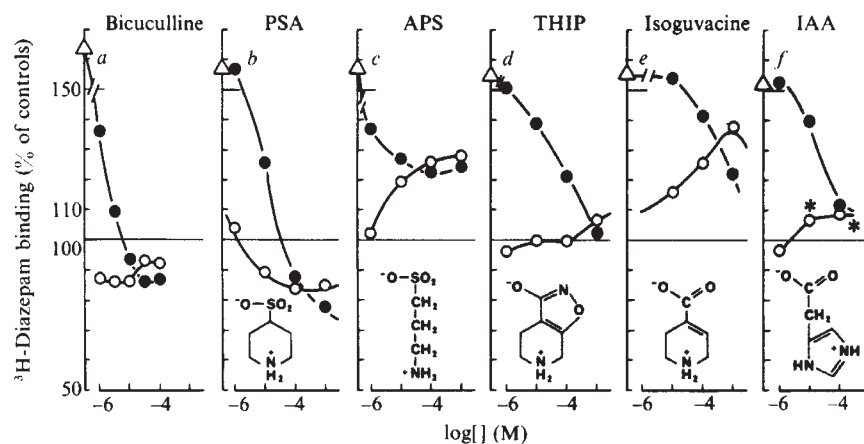


Fig. 1 Effects of bicuculline and some potent GABA-mimetics on ^3H -diazepam binding to rat brain membranes. $\circ$, GABA-mimetic alone; $\bullet$, GABA-mimetic plus 3×10^{-6} M muscimol; $\triangle$, muscimol alone, 3×10^{-6} M. Whole rat forebrains were homogenised in 10 vol ice-cold 100 mM Tris-citrate pH 7.1, in a precooled Ultra-Turrax homogeniser. The homogenate was centrifuged at 48,000g for 10 min at $0-4^\circ\text{C}$ and the pellet was rehomogenised for a few seconds in another 10 vol of the same ice-cold buffer. Centrifugation and rehomogenisation were performed five times. The final suspension was diluted to 160 vol (vol/weight original tissue) in 100 mM Tris-citrate pH 7.1, and aliquots of 2.5 ml were incubated with 0.4 nM ^3H -diazepam ($14.4 \text{ Ci mmol}^{-1}$, gift from Willy Haefely, Roche) for 20 min at 0°C in a total of 2.75 ml.

GABA antagonists and agonists were added in duplicate in that order just before ^3H -diazepam. Samples were diluted to 10 ml in cold buffer after incubation and filtered immediately through GF/C glass-fibre filters and washed with 10 ml buffer. Tritium on filters was assessed by scintillation counting. All binding values were calculated as specific binding, which is total binding minus binding in the presence of 3×10^{-6} M unlabelled diazepam. Control specific binding averaged 677 ± 49 c.p.m. per 15 mg tissue; specific binding in the presence of 3×10^{-6} M muscimol, $1,037 \pm 70$ c.p.m. per 15 mg tissue; and nonspecific binding 157 ± 15 c.p.m. per 15 mg tissue (mean $\pm$ s.e.m. of six experiments). The data shown are from a single experiment which was repeated giving similar results. Bicuculline (Sigma) was freshly prepared in dilute HCl; piperidine-4-sulphonic acid and THIP, H_2O were synthesised by P.K.-L.; APS (sodium salt) was supplied by J. Arnt, Copenhagen; isoguvacine, HCl was synthesised by O. Rosenlund-Hansen, Ferrosan. * Imidazoleacetic acid (10^{-5} – 10^{-4} M) increased ^3H -diazepam binding ($17.6 \pm 2.04\%$ in six experiments, $P < 0.001$).

Activation of benzodiazepine receptors by GABA is achieved at micromolar concentrations, as are GABA effects on the superior cervical ganglion¹⁰, the cuneate nucleus *in vitro*¹¹, the crayfish stretch receptor neurone¹² and the dorsal root ganglion neurone¹³. (3) The only non-GABA-agonist (potassium iodide, 30 mM, see also ref. 14) among many tested which increased ^3H -diazepam binding in the present membrane preparation acts in an additive way to muscimol, suggesting a different mechanism of action⁴.

A second group of potent GABA-mimetics, piperidine-4-sulphonic acid, APS, IAA, THIP and isoguvacine (see below) did not, however, increase ^3H -diazepam binding to any great extent (Fig. 1b–f), even at concentrations more than 10,000-fold in excess of the respective IC_{50} values^{6,7} for ^3H -GABA binding (see also refs 2, 15). These compounds surprisingly inhibited muscimol activation to a degree depending on their respective efficacies (Fig. 1b–f). Piperidine-4-sulphonic acid also completely inhibited the activation induced by *trans*-amino-crotonic acid and GABA. GABA analogues of restricted conformation (isomuscimol, iso-THIP and homologues of THIP with seven-membered heterocyclic rings⁶) were inactive as inhibitors of ^3H -GABA binding, as activators of ^3H -diazepam binding and as inhibitors of muscimol activation.

Parallel displacement of dose–response curves for muscimol and linear Schild plots with slopes close to unity (Fig. 2) indicate that piperidine-4-sulphonic acid is a competitive inhibitor of muscimol in a first-order interaction. If piperidine-4-sulphonic acid had been a pure agonist to GABA receptors, an EC_{50} value of $179 \times \text{IC}_{50} = 7.1 \mu\text{M}$ would be expected ($\text{IC}_{50} = 40 \text{ nM}$ for ^3H -GABA binding, P.K.-L. unpublished). The antagonist affinity, $K_1 = 1.5 \mu\text{M}$ (Fig. 2b), is relatively close to this predicted value, indicating that piperidine-4-sulphonic acid occupies GABA receptors with the expected affinity but without efficacy in the present system. Piperidine-4-sulphonic acid and THIP are antagonists in the present membrane preparation (washed membranes). Partial agonist properties cannot be excluded since both compounds exhibit marginal agonistic effects in Triton-treated membranes⁴.

Piperidine-4-sulphonic acid (P.K.-L., D. R. Curtis and E.F. unpublished), APS¹⁶, IAA^{6,17}, THIP^{6,18} and isoguvacine^{6,18} are GABA-mimetic in the sense that they inhibit high-affinity GABA receptor binding with IC_{50} values ranging from 6 to 130 nM, and that they are potent bicuculline-sensitive inhibitors of cat spinal cord motoneurons. However, when tested on the coupled GABA-receptor benzodiazepine receptor *in vitro*, these compounds exhibit mixed agonist/antagonist or pure

antagonist properties. The physiological relevance of this feature is unclear, but the finding that APS is less active on crayfish stretch receptor neurones than expected from receptor binding studies¹⁹, and the unexpected lack of GABA-like activity of THIP and isoguvacine on E4 cells in the visceral ganglion of *Helix aspersa*²⁰ may be a consequence of low efficacy.

It is thus conceivable that several classes of GABA receptors exist in the central nervous system; some (those coupled to benzodiazepine receptors) may differentiate a continuum of pure and partial agonists, whereas other classes possess no such selectivity.

The experiments described here introduce a simple technique for studying 'classical' pharmacology in the central nervous system. Other high-affinity binding sites may be coupled, making the principle more generally applicable.

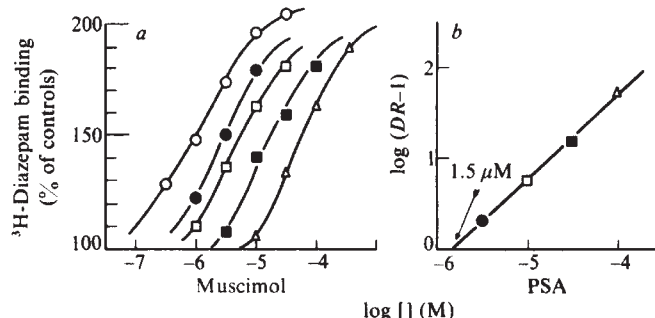


Fig. 2 a, Dose–response curves for the effect of muscimol on ^3H -diazepam binding to rat brain membranes prepared as described in Fig. 1. Various concentrations of piperidine-4-sulphonic acid were added. $\circ$, No addition; $\bullet$, 3×10^{-6} M; $\square$, 10^{-5} M; $\blacksquare$, 3×10^{-5} M; $\triangle$, 10^{-4} M. b, Schild plots constructed from the EC_{50} values for muscimol obtained in a. (The dose–response ratio, DR , is the ratio of EC_{50} values for muscimol with and without antagonist added.) Slope in b, 0.92. The EC_{50} value is that concentration of GABA agonist in μM which increases specific ^3H -diazepam binding to half the maximal increase obtainable with optimal concentrations (10^{-5} – 3×10^{-5} M) of muscimol. EC_{50} values given are means of at least two values, each obtained by Hill analysis of results obtained by addition of four to ten concentrations of agonist in duplicate assays. The results shown in this figure are from a single experiment which was repeated twice with similar results. Each point in a is the mean of two values which differed $2.7 \pm 0.5\%$ (25); the control level was 784 ± 15 c.p.m. per 15 mg tissue (9) (mean $\pm$ s.e.m. of (n) values).

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1. Squires, R. F. & Braestrup, C. *Nature* **266**, 732-734 (1977).
2. Tallman, J. F., Thomas, J. W. & Gallager, D. W. *Nature* **274**, 383-385 (1978).
3. Haefely, W. et al. in *GABA-Neurotransmitters, Pharmacological, Biochemical and Pharmacological Aspects* (eds Krosgaard-Larsen, P., Scheel-Krüger, J. & Kofod, H.) 357-375 (Munksgaard, Copenhagen, 1979).
4. Braestrup, C., Nielsen, M., Krosgaard-Larsen, P. & Falch, E. in *Receptors for Neurotransmitters and Peptide Hormones* (eds Kuhar, M., Enna, S. & Pepeu, G.) (Raven, New York, in the press).
5. Karobath, M., Placheta, P., Lippitsch, M. & Krosgaard-Larsen, P. *Nature* **278**, 748-749 (1979).
6. Krosgaard-Larsen, P., Honore, T. & Thyssen, K. in *GABA-neurotransmitters, Pharmacological, Biochemical and Pharmacological Aspects* (eds Krosgaard-Larsen, P., Scheel-Krüger, J. & Kofod, H.) 201-216 (Munksgaard, Copenhagen, 1979).
7. Krosgaard-Larsen, P., Hjeds, H., Curtis, D. R., Lodge, D. & Johnston, G. A. R. *J. Neurochem.* (in the press).
8. Wastek, G. J., Speth, R. C., Reisine, T. D. & Yamamura, H. I. *Eur. J. Pharmac.* **50**, 445-447 (1978).
9. Gallager, D. W., Thomas, J. W. & Tallman, J. F. *Biochem. Pharmac.* **27**, 2745-2750 (1978).
10. Bowery, N. G. & Brown, D. A. *Br. J. Pharmac.* **50**, 205-218 (1974).
11. Simmonds, M. A. *Br. J. Pharmac.* **63**, 495-502 (1978).
12. Hori, N., Ikeda, K. & Roberts, E. *Brain Res.* **141**, 364-370 (1978).
13. Deschenes, M. & Feltz, P. *Brain Res.* **118**, 494-499 (1976).
14. Costa, T., Rodbard, D. & Pert, C. B. *Nature* **277**, 315-317 (1978).
15. Karobath, M. & Sperk, G. *Proc. natn. Acad. Sci. U.S.A.* **76**, 1004-1006 (1979).
16. Beaumont, K., Chilton, W. S., Yamamura, H. I. & Enna, J. *Brain Res.* **148**, 153-162 (1978).
17. Curtis, D. R., Duggan, A. W., Felix, D. & Johnston, G. A. R. *Brain Res.* **32**, 69-96 (1971).
18. Krosgaard-Larsen, P., Johnston, G. A. R., Lodge, D. & Curtis, D. R. *Nature* **268**, 53-55 (1977).
19. Roberts, E., Wong, E., Krause, D. N., Ikeda, K. & Degener, P. in *GABA-neurotransmitters, Pharmacological, Biochemical and Pharmacological Aspects* (eds Krosgaard-Larsen, P., Scheel-Krüger, J. & Kofod, H.) 46-59 (Munksgaard, Copenhagen, 1979).
20. James, V. A., Krosgaard-Larsen, P. & Walker, R. J. *Experientia* **34**, 1630-1631 (1978).

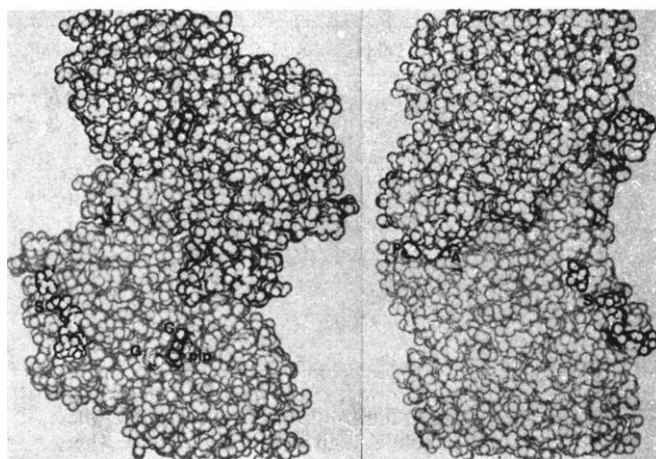


Fig. 1 Two orthogonal views of the Van der Waals contact surface of the phosphorylase *a* dimer. All nonhydrogen atoms at the surface are shown as well as several ligands which bind to the enzyme: S, maltoheptaose; G, a buried glucose molecule; C, caffeine; plp, the buried coenzyme pyridoxal phosphate; P, the serine-14-phosphate and A, AMP. The atomic positions were determined by X-ray diffraction at 2.5 Å resolution. The two subunits are shown in different shades of grey. Note that although the dimer contact appears to be extensive in these drawings, a large solvent accessible cavity of about 2,500 Å³ lies at the subunit interface. The left plate shows the face of the dimer that binds to glycogen¹¹. This same face is seen edge-on at the right in the orthogonal view.

globular shape for which the surface area/volume ratio approaches the minimum value characteristic of a sphere. (It is not implied that proteins are necessarily spherical.) Interpenetrating spherical surfaces have been used to model the accessible surface changes in the dimerisation of insulin, the interaction between haemoglobin α and β subunits and of trypsin with its inhibitor³.

Using an extended data base, Janin⁴ has recently shown that the number of buried ($A_b < 20$ Å² per residue) amino acids, n_b , in a protein of n residues is given by

$$n_b = (n^{1/3} - k)^3 \quad (2)$$

where $k = 1.62$.

As many proteins of biological interest are large, multidomain and oligomeric, it is of interest to determine the applicability of models based on simpler structures to these more complex systems. We have recently determined the atomic coordinate set for rabbit muscle phosphorylase *a* (ref. 5). This protein has about three times more amino acids per polypeptide chain than the largest used to determine the accessibility law. Further, phosphorylase occurs as a dimer in the tetragonal crystal form discussed here, allowing a determination of the protein surface removed from solvent contact by dimerisation. Using a program written by T. J. Richmond, incorporating the algorithm of Lee and Richards¹, we have calculated the solvent-accessible surface areas for monomers and dimers of phosphorylase *a*, chicken triose phosphate isomerase (TIM), horse alcohol dehydrogenase (ADH), human Bence-Jones protein (BJ) and the monomer and tetramer of dogfish apolactate dehydrogenase (LDH). Atomic coordinates were obtained from the AMSOM protein data files⁶. Accessible surface areas and related parameters are summarised and described in Table 1. The data for insulin and the haemoglobin α and β chains given by Chothia and Janin⁹, which agree with our calculations for these cases, are included for comparison.

It seems that expression (1) holds well for low molecular weight protein monomers. However, for larger proteins, the model underestimates accessible surface area by a margin of error which increases with the molecular weight of the monomer. This discrepancy has already been noted for oligomeric proteins^{3,4}. The model errs most seriously with phosphorylase.

Solvent accessibility properties of complex proteins

AN important factor in the thermodynamics of protein folding is the partition of amino acid residues between the solvent environment and the protein core. The extent of the interaction between the folded polypeptide and the surrounding medium can be measured by determining the solvent accessible surface area as defined by Lee and Richards¹. In this communication we present the results of solvent accessibility calculations for the large (195,000 M_r) dimeric enzyme phosphorylase *a* and other multidomain, multisubunit proteins. These results are discussed in the light of the generalisations concerning protein solvent-accessible surface area and shape which have been based on data derived from low molecular weight proteins.

X-ray diffraction studies of proteins have, in many cases, provided atomic coordinates of sufficient accuracy to make accessibility calculations feasible. Using the crystallographically-derived coordinates for a select group of small ($M_r \leq 30,000$), compact, well determined protein structures, Janin² and Teller³ have demonstrated that the solvent-accessible surface area, A_s , can be expressed as a function of molecular weight:

$$A_s = 11.1 M^{2/3} \quad (1)$$

The standard error between calculated and theoretical values of A_s , for structures incorporated in the data base is about 400 Å² (ref. 3). This model predicts that protein molecules adopt a

Table 1 Predicted and observed solvent-accessible surface areas of oligomeric proteins

Protein	Molecular weight	Monomer					Oligomer			Interface	
		$A_s^{obs} (\text{\AA}^2)$	A_s^{th}	F_{as}^{obs}	n_B	n_B^{th}	A_s^{obs}	A_s^{th}	F_{as}^{obs}	F_B^{obs}	F_B^{th}
Insulin [†]	5800	3440	3580	0.59	—	—	5580	5900	0.67	0.17	0.14
Bence-Jones	11700	5590	5730	0.67	33	30	9490	9820	0.72	0.13	0.14
Haemoglobin α^+	15100	6940	6800	0.68	—	—	12320	11870	0.72	0.12	0.15
β	16000	7130	7050	0.69	—	—					
TIM	26500	11450	9870	0.70	100	84	19730	16920	0.74	0.14	0.14
LDH	36400	17440	12210	0.66	114	134	47150	34870	0.78	0.32	0.28
ADH	39700	16530	12930	0.71	159	174	29720	22160	0.74	0.10	0.14
Phosphorylase	97400	36400	23530	0.74	357	478	68200	40330	0.76	0.06	0.14

* Derived quantities: A_s^{th} , solvent accessibility calculated from equation (1); for dimers and the tetramer of LDH accessible surface area is given by the expression $2 \times (0.86 A_s^{th})$ and $4 \times (0.72 A_s^{th})$, respectively, where A_s^{th} is the surface area of the monomer³. F_{as} , the fraction of potentially accessible surface buried by protein folding is given by $(A_s - A_s^{obs})/A_s$ where $A_s (= 1.44 M)$ is the accessible area of an extended polypeptide chain⁷. n_B is the number of residues buried by protein folding predicted by equation (2). F_B^{obs} and F_B^{th} are the observed and predicted³ values for solvent-accessible surface area buried at the dimer or tetramer interface.

[†] Reproduced from ref. 3.

The expression proposed to predict n_B (equation (2)) is reasonably accurate for most of the cases listed in Table 1 (only monomers are considered), but does not apply for monomers of LDH and phosphorylase. The sphere interpenetration model for dimerisation predicts that approximately 14% and 28% of the monomer surface is buried after the formation of dimers and square planar tetramers, respectively³. It is interesting that theoretical values of A_s for the TIM dimer and the LDH tetramer prove to be more accurate predictions than the theoretical values of A_s for the isolated monomers. A minimum surface model seems to fit the oligomers better than their respective monomers. Phosphorylase is again a notable exception. Only 6% of the accessible surface of the monomer (2,300 $\text{\AA}^2$) is involved in dimer formation. This analysis does not account for possible conformational changes on oligomerisation (see below).

Although minor deviations in observed A_s and n_B from values predicted by equations (1) and (2) may be attributed to non-spherical shape, this explanation cannot account for the significant underestimation for phosphorylase. For example, one can readily show that a parallelepiped of dimensions sufficient to completely enclose the phosphorylase monomer would have a surface area equal to that of the protein but would contain twice its volume. In fact, the large accessible areas of LDH, ADH and phosphorylase are most probably due to the articulation of each of these molecules into two well-defined functional and structural domains, which gives rise to a complex and invaginated molecular topography. This is illustrated in the space-filling model of the phosphorylase dimer (Fig. 1). Chothia⁷ has suggested that in such large multidomain or multi-subunit structures, the fraction of potentially accessible surface area buried by protein folding, F_{as} , is essentially constant. Table 1 shows that F_{as} for monomers and multimers becomes asymptotic at 0.75–0.78. This implies that the accessible surface becomes a linear function of M_r for large proteins. A plot of A_s against M_r from Table 1 shows this to be true.

Although only a small fraction of the phosphorylase molecule is involved in dimer formation, the amount of surface area withdrawn from solvent by formation of the complex is quite large, 4,600 $\text{\AA}^2$. Loss of accessible surface area is believed to contribute significantly to the free energy of protein association^{8,10} which may be expressed (for dissociation) as

$$\Delta H_d + \Delta G_d' - \Delta G_d^s = \Delta G_d \quad (3)$$

where ΔG_d^s and $\Delta G_d'$ are free energy terms due, respectively, to the gain in rotational and translational entropy by the subunits upon dissociation and to the entropy lost by solvent as a result of the increase in subunit accessible area. The enthalpy of dissociation, ΔH_d , is assumed to be small^{8,9} due to the energetic equivalence between solvent-monomer and monomer-monomer hydrogen bonds and Van der Waals' interactions. The free energy of dissociation, ΔG_d , is $-RT \ln K_d$. These

parameters may be evaluated for the dissociation of the phosphorylase dimer.

Using the expression derived from statistical thermodynamics by Janin¹⁰, ΔG_d^s is 33 kcal mol⁻¹. The hydrophobic free energy $\Delta G_d'$ has been given as a direct function of the change in accessible surface area ($\gamma \Delta A_s$, where $\gamma \sim 25$ cal mol⁻¹ $\text{\AA}^2$)^{8,9}. Assuming $\Delta A_s \sim 2A_{s, \text{monomer}} - A_{s, \text{dimer}}$, $\Delta G_d'$ is approximately 155 kcal mol⁻¹. Substituting these values into equation (3), we arrive at the clearly unreasonable estimate of 82 kcal mol⁻¹ ($K_d \sim 10^{-60}$) for ΔG_d . If only nonpolar atoms, that is, carbon atoms, are considered in the accessibility calculation, ΔA_s is about 2,600 $\text{\AA}^2$, corresponding to $\Delta G_d'$ of 33 kcal mol⁻¹ ($K_d \sim 10^{-24}$). Preliminary kinetic studies (N.B. Madsen, personal communication) indicate ΔG_d for phosphorylase is probably about 11 kcal mol⁻¹.

It is unlikely that errors in the phosphorylase coordinate set can account for these discrepancies. One source of error could be the assumption that the subunit assumes the same conformation within the dimer as it does as a monomer. For example, the free monomer may bury a significant proportion of the accessible surface area which is also observed to be buried in the dimer. If this were indeed the case, we would conclude (assuming $\Delta H_d \sim 0$ and $\Delta G_d \sim 11$ kcal mol⁻¹) from equation (3) that ΔA_d is only 900 $\text{\AA}^2$ per monomer. Another source of error could be the assumption of $\Delta H_d \approx 0$. The enthalpy, if non-zero,

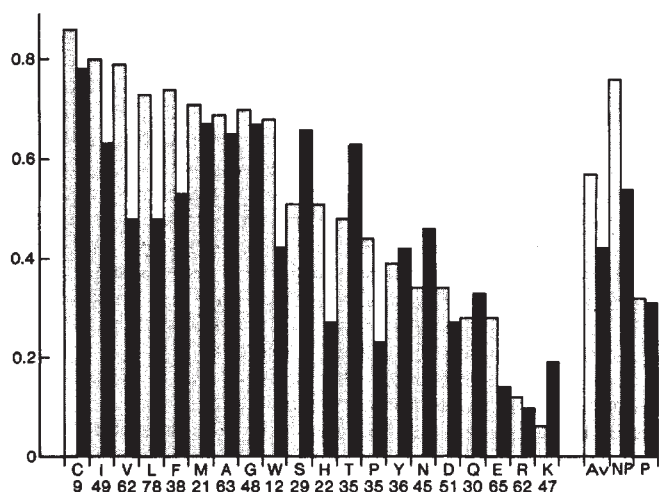


Fig. 2 The ordinate shows the mole-fraction buried for each type of amino acid and the distribution predicted using the partition coefficients determined by Janin⁴. The number of each type of amino acid is shown below the usual one letter code representation. The three additional entries at the right represent respectively the average over all amino acids (Av) the nonpolar amino acids (Ile, Val, Leu, Phe, Met, Ala, Trp, Gly, Pro; NP) and the polar amino acids (P).

could contain contributions from subunit association and/or subunit conformational changes.

As the bulk solvent accessibility properties of phosphorylase differ significantly from those predicted from smaller systems, it is of interest to note the relative accessibility of polar and nonpolar side chains. Chothia² has found that the proportion of non-polar surface buried increases with molecular weight, whereas the proportion of polar surface buried is constant. A similar conclusion is drawn from Janin's results⁴. Figure 2 shows the mole fraction buried for each of the amino acids in phosphorylase compared with that predicted for phosphorylase using partition coefficients determined by Janin⁴ for each residue, and n_b as observed (357) (see Table 1 and equation (3) in this reference). Generally fewer of the nonpolar residues (Ile, Val, Leu, Phe, Trp and Pro) are buried in phosphorylase than predicted from the data base; the accessibility for the polar residues (Ser, Thr, Asn, Gln and Lys) is lower than predicted. (Note that there is relatively little difficulty with sample size in these statistics since only Trp and Cys appear fewer than 20 times.)

Accounting for these deviations from predicted accessible surface in the case of phosphorylase is difficult. It is unlikely on a biochemical basis that other macromolecules, for example, kinase, phosphatase and glycogen, bury additional surface *in vivo*. The most likely explanation for the discrepancy in accessible surface and the distribution of buried residues is that the data base used for deriving expression (1) rests on a small sample of mainly extracellular proteins of low molecular weight. These proteins are generally required by their environment to be structurally more stable than the large and conformationally labile intracellular regulatory proteins, such as phosphorylase. We feel that the closing statement of Lee and Richards' original paper¹ is still appropriate: "Simple generalisations of any sort are remarkably difficult to see in the summaries so far produced".

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1. Lee, B. & Richards, F. M. *J. molec. Biol.* **55**, 379–400 (1971).
2. Chothia, C. *J. molec. Biol.* **105**, 1–14, Appendix, Janin, J. (1976).
3. Teller, D. C. *Nature* **260**, 729–731 (1976).
4. Janin, J. *Nature* **277**, 491–492 (1979).
5. Sprang, S. R. & Fletterick, R. J. *J. molec. Biol.* (in press).
6. Feldman, R. J. *Atlas of Macromolecular Structure on Microfiche* (Tracor Jitco, Rockville, Maryland, 1976).
7. Chothia, C. *Nature* **254**, 304–308 (1975).
8. Chothia, C. *Nature* **248**, 338–339 (1974).
9. Chothia, C. & Janin, J. *Nature* **256**, 705–708 (1975).
10. Janin, J. *Biochemistry* **17**, 2947–2948 (1978).
11. Fletterick, R. J., Sprang, S. R. & Madsen, N. B. *Can. J. Biochem.* (in press).

Evidence for non-spliced SV40 RNA in undifferentiated murine teratocarcinoma stem cells

UNDIFFERENTIATED murine teratocarcinoma stem cells do not support simian vacuolating virus 40 (SV40) or polyoma virus (Py) replication or even the expression of the early papovavirus proteins. These undifferentiated cells are also entirely refractory to infection with ecotropic murine C-type viruses. If the stem cells are allowed to differentiate to a variety of somatic cell types, they become susceptible to infection by SV40, Py and ecotropic murine C-type viruses^{1–4}. In contrast, adenovirus type

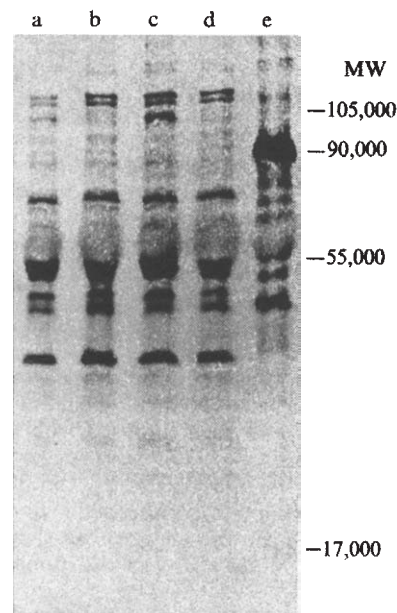


Fig. 1 Analysis of proteins in SV40-infected F-9 cells. Cultures of 1×10^7 F-9 cells uninfected or infected with SV40 (250 p.f.u. per cell) for 17 h, were labelled for 2 h with ^{35}S -methionine ($100 \mu\text{Ci ml}^{-1}$) in methionine-free medium. Proteins were purified and immunoprecipitated with hamster anti-SV40 T-serum as described previously¹⁰. Samples were electrophoresed in 20% polyacrylamide slab gels (20% acrylamide–0.1% bisacrylamide) for 10 h at 75 V (ref. 11). The autoradiograph shows proteins extracted from uninfected F-9 cells immunoprecipitated with anti-SV40 T-serum (a); or with normal hamster serum (b); proteins extracted from SV40-infected F-9 cells immunoprecipitated with anti-SV40 T-serum (c); or with normal hamster serum (d); control proteins from SV40-infected AGMK cells immunoprecipitated with anti-SV40 T-serum (e).

2 can infect and replicate in the undifferentiated stem cells, although not as efficiently as it can in the differentiated mouse cells⁵ and certainly much less efficiently than it does in human cells. Studies on the mechanism of the resistance of the undifferentiated teratocarcinoma cells to infection by SV40 has shown that the block is not at the level of virus adsorption, penetration uncoating or transport to the nucleus⁶. We present here evidence indicating that the absence of expression of SV40 genetic information in nondifferentiated teratocarcinoma cells reflects an inability to generate stable viral mRNA. More specifically, the refractoriness of these cells to SV40 seems to be correlated, at least in part, with a deficiency in the splicing of viral mRNA.

The F-9 line of mouse teratocarcinoma cells, a nullipotent stem cell line that does not differentiate in normal culture conditions^{7–9}, was used. Cells were generally inoculated with high multiplicities of infection (MOI) of plaque-purified wild-type SV40 (50–250 plaque-forming units (p.f.u.) per cell). Although infection of differentiated mouse cells (for example primary mouse embryo or 3T3 cells) with SV40 does not lead to the synthesis of viral DNA or the production of late viral polypeptides, substantial amounts of SV40 large T- and small t-antigens and the early SV40 RNA which encodes these proteins can usually be detected. In contrast, others have shown that SV40 infection of undifferentiated teratocarcinoma cells does not result in detectable T-antigen synthesis¹, as assayed by immunofluorescence. To confirm this observation we examined the SV40-infected F-9 cells for SV40 antigens using the more sensitive technique of immunoprecipitation and polyacrylamide gel electrophoresis (PAGE). After labelling SV40-infected F-9 cells with ^{35}S -methionine 17 h post-infection (p.i.), proteins in the soluble extracts were immunoprecipitated either with a T-serum (obtained from hamsters bearing SV40 tumours), or with a control normal hamster serum¹⁰. The immunoprecipitated proteins were analysed on 20% polyacrylamide-SDS

gels¹¹. As shown in Fig. 1c, SV40-infected F-9 cells do not synthesise either of the known early SV40 gene products (90,000-MW T-antigen and 17,000-MW t-antigen^{12,13}). However, two distinct polypeptides with apparent molecular weights of 105,000 and 55,000 were immunoprecipitated with anti-SV40 T serum, both from the SV40-infected and from the uninfected F-9 cells (Fig. 1a,c). These two proteins were not seen in the control reactions with normal serum (Fig. 1b,d), suggesting that although anti-T serum has antibodies against the 105,000 and 55,000-MW proteins, these may not be encoded by SV40. Further analysis of these two proteins will be presented elsewhere. It has been previously shown^{14,15} that an antiserum from rabbits which had been immunised with cells from the embryoid bodies of mouse teratocarcinomas contain several distinguishable antibody specificities, including an activity against Py- and SV40-transformed mouse fibroblast lines and a variety of mouse tumour cells. Our results suggest that the two immunoprecipitable proteins (105,000 and 55,000 MW) may represent common polypeptides synthesised in undifferentiated and SV40-transformed mouse cells. The significance and potential relationship of these proteins to tumorigenesis are being investigated.

As we were unable to detect SV40 T-antigens in the infected F-9 cells, we sought to determine whether the inoculated SV40 DNA was being transcribed. From previous studies¹⁶ it was clear that the most sensitive assay for detection of viral transcription is the *in vitro* sarkosyl method¹⁷. This technique relies on the fact that viral transcriptional complexes (VTCs) which are initiated *in vivo* can be extended (but not initiated) *in vitro*; its sensitivity derives from: (1) the ability to incorporate high specific activity precursors into growing RNA chains; (2) removal of potential processing enzymes which could degrade 'unstable' RNA species; and (3) separation of the viral transcriptional activity in the sarkosyl supernatant fraction from the bulk of the cellular transcriptional activity which resides in chromatin pellet. Ten hours after infection of F-9 cells (MOI = 250) VTCs were isolated and incubated in a standard reaction mixture¹⁸ containing [α -³²P]ATP. (In a separate experiment (S.S., E. May and G.K., in preparation), 10 h p.i. was found to correspond to the peak of viral transcriptional activity in mouse cells.) RNA was purified and annealed with the separated strands of two SV40 DNA fragments (representing early and late regions of the viral genome) which had been immobilised on nitrocellulose filters¹⁹ (Fig. 2). The pattern of hybridisation clearly indicates that viral transcription is initiated in SV40-infected F-9 cells. As the transcriptional activity can be detected in the supernatant fraction, this transcription seems to be initiated, at least in part, on free viral DNA molecules¹⁸. Finally, the ratio of early strand to late strand transcription (5.5:4.5), determined by a densito-

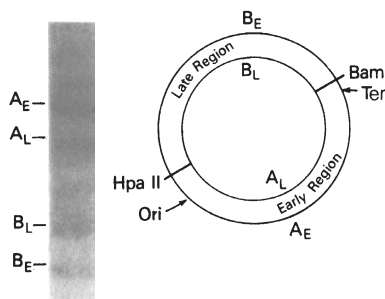


Fig. 2 Analysis of *in vitro*-extended RNA from SV40-infected F-9 cells. Viral transcriptional complexes (VTCs) were prepared using the sarkosyl extraction method¹⁷ from the nuclei of SV40-infected F-9 cells 10 h after infection. After *in vitro* incorporation of [α -³²P]ATP (350 Ci mmol⁻¹) into the elongating RNA chains¹⁶, RNA was isolated as previously described¹⁸. Strand orientation and genomic region of the RNA was determined by hybridisation to the separated strands of *Bam*HI/*Hpa*II-cleaved SV40 DNA immobilised on nitrocellulose filter¹⁹. Ori, origin of viral DNA replication; Ter, terminus of DNA replication (these sites divide SV40 into the early and late gene regions); A_E = SV40 early coding strand; B_E, early non-coding; B_L, late coding; A_L, late noncoding.

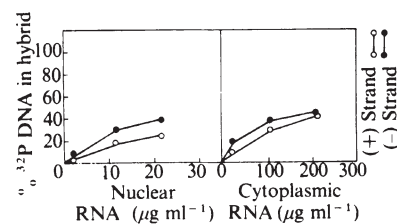


Fig. 3 Analysis of unlabelled *in vivo* viral RNA from SV40-infected F-9 cells. Nuclear and cytoplasmic RNAs were prepared from SV40-infected F-9 cells (250 p.f.u. per cell) 10 h p.i. as described elsewhere²³. Increasing concentrations of RNA (cytoplasmic and nuclear) were hybridised for 20 h to fragmented ³²P-labelled separated single strands of SV40 DNA (specific activity, 1×10^6 c.p.m. μ g⁻¹) in conditions previously described²⁷; the fraction of SV40 L and E strands in hybrid molecules was determined by hydroxyapatite chromatography²⁰.

metric tracing of the autoradiograms, indicates that substantial fractions of viral transcription are initiated on both early and late viral DNA strands. Although it is difficult to quantitate the *in vitro* transcriptional activity specifically, similar amounts of virus-specific RNA have been obtained from VTCs of differentiated and undifferentiated mouse cells (S.S. *et al.*, in preparation).

We consequently undertook a second set of experiments in an attempt to detect and characterise viral RNA transcribed *in vivo*. Unlabelled cytoplasmic and nuclear RNAs were purified 10 h after infection of F-9 cells. To determine the proportion of the SV40 genome that is transcribed *in vivo*, increasing concentrations of the cytoplasmic and nuclear RNAs were annealed in solution with separated strands of ³²P-labelled single-strand fragments of SV40 DNA. The fraction of each DNA strand in hybrid molecules was determined by hydroxyapatite chromatography²⁰. As seen in Fig. 3, the extent of hybridisation between the (-)strand and (+)strand of SV40 DNA and increasing RNA concentrations was almost linear and did not reach a plateau. At the highest RNA concentration used, about 40% of the (-)strand and 20% of the (+)strand fragments appear in duplex molecules. This result supports the VTC study in showing that both early and late strands of SV40 are transcribed in F-9 cells, although the fraction of each strand represented in RNA is not established by this experiment. The paucity of stable SV40 RNAs in F-9 cells prompted a further analysis of these transcripts. As early SV40 RNA seems to be more abundant than late RNA in F-9 cells and only the early transcripts are translated into detectable virus-specific proteins in mouse embryo or 3T3 cells, we focused on an analysis of the early viral RNA. Berk and Sharp have recently introduced an alkaline agarose gel electrophoresis technique which can effectively size and map both co-linear and spliced RNA molecules²¹. ³²P-double-stranded DNA probes are annealed with RNA in conditions which favour DNA-RNA annealing. The hybrid species are treated with S₁ nuclease before electrophoresis and the labelled DNA, which can be accurately sized as single-stranded molecules, provides a direct representation of the RNA molecules present. This method has demonstrated the presence of two spliced early SV40 mRNAs which code for T and t antigens²² (see Fig. 4a). We have recently found that the sensitivity of this technique can be amplified if one uses strand-separated DNA probes, thus permitting hybridisation in much less stringent conditions. This modification is crucial to an analysis of transcripts as scarce as those present in SV40-infected F-9 cells.

Both total RNA and cytoplasmic RNA were isolated 10 h after SV40 infection of either differentiated primary mouse embryo (ME) cells or the undifferentiated F-9 teratocarcinoma cells. The cytoplasmic ME and F-9 RNAs were further fractionated into poly(A)-containing (poly(A)⁺) and non-poly(A)-containing (poly(A)⁻) fractions. For a probe, ³²P-labelled SV40 DNA was cleaved with *Bam*HI and *Hpa*II restriction endonucleases and the early coding single strand of the DNA

fragment mapping from 0.145 to 0.725 (*Bam*HI/*Hpa*II A_E) was purified¹⁹ and hybridised with the RNAs described above. After annealing, hybrid molecules were digested with nuclease S₁ and the products were analysed by alkaline agarose gel electrophoresis.

In a control experiment (Fig. 4a), the S₁ technique was used to map early SV40 cytoplasmic RNAs from lytically infected monkey kidney (MK) cells. As expected, three bands were observed which measured 0.38, 0.12 and 0.065 SV40 map units. These represent the RNA body coding for T and t antigens, (0.38) and the (0.12) and (0.065) leader segments (Fig. 4c).

In contrast, (Fig. 4b) the hybridisation of nuclear or cytoplasmic early RNA from SV40-infected F-9 cells produced one band migrating at a position corresponding to 0.52 SV40 units length. This fragment has been positioned at between 0.67 and 0.15 map units and corresponds directly in size and map position with the primary early SV40 transcript found in the nuclei of SV40-infected MK cells (unpublished data). The analysis of RNA from SV40-infected differentiated primary mouse embryo cells is also shown in Fig. 4b. Total cellular RNA from SV40-infected ME cells produced two major bands, one corresponding to a non-spliced early SV40 transcript (0.52 SV40 map units) and the second representing the body of the early mRNAs (0.38 SV40 map units). Poly(A)⁻ RNA contained only the band of the nonspliced RNA while poly(A)⁺ cytoplasmic RNA contained one band corresponding to the processed early mRNA body. This finding supports previous studies which indicated that polyadenylation of RNA precedes splicing^{23,24}. The t (0.12 map units) and T (0.065 map units) leader segments have been run off

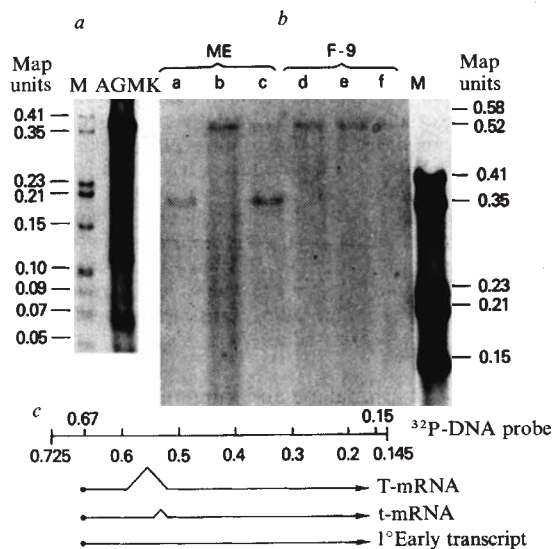


Fig. 4 Sizing of transcripts from SV40 infected mouse embryo (ME) and F-9 embryonal carcinoma cells. Total cellular RNA, poly(A)⁻ and poly(A)⁺ cytoplasmic RNAs were extracted from ME and F-9 cells, 10 h after infection with SV40 (250 p.f.u. per cell). These RNAs were separately annealed with a single-stranded ³²P-SV40 DNA probe (1.5 × 10⁶ c.p.m. μg⁻¹) extending from map units 0.145 to 0.725 and representing the entire early region of SV40. Preparation of this probe has been described in detail¹⁹. After annealing for 18 h at 37°C in 50% formamide, 0.4 M NaCl, 0.04 M PIPES buffer (pH 6.8), hybrid molecules were digested with an excess of nuclease S₁ (45°C for 30 min) and analysed by alkaline agarose gel electrophoresis²¹. The products of S₁ analysis of hybrids between the early strand probe and poly(A)⁺ cytoplasmic RNA from SV40-infected permissive MK cells are a control (a). Three prominent bands are evident representing the splicons for T- and t-mRNAs (0.065 and 0.12 map units) as well as their 3'-body segment (0.38 map units). b, S₁ products of hybrids between the same DNA probe and RNA from SV40-infected ME (a,b,c) or F-9 cells (d,e,f). The RNA preparations included poly(A)⁺ cytoplasmic RNA (a,d) poly(A)⁻ total cellular RNA (b,e) and total infected cell RNA (c,f). M, DNA size markers. Map units are given as fractional lengths of the SV40 genome. c, The two splices SV40 lytic mRNAs (encoding T and t peptides) and primary early transcript from which they are thought to be processed.

this gel to obtain a better resolution of the larger bands. The results of this experiment indicate that a spliced early mRNA is synthesised during the infection of differentiated mouse embryo cells by SV40. The detection of the full early transcript in the total cell (nuclear-plus cytoplasmic) RNA as well as the poly(A)⁻ RNA suggests that the mature cytoplasmic spliced RNAs are derived from this primary transcript. Of particular importance is the detection of the primary transcript alone in the infected F-9 cells. This finding suggests that a major block to SV40 gene expression in F-9 cells is related to the inability to splice the primary transcripts. We cannot exclude, however, the possibility that an additional step in SV40 gene expression, for example the initiation of SV40 transcription, is also depressed in these cells. Nor can we exclude the possibility that other embryonal carcinoma cell lines may have other restrictions to SV40 gene expression. In contrast, spliced early SV40 mRNAs are efficiently produced in differentiated mouse embryo cells. Recent experiments using SV40 deletion mutants^{16,25,26} and recombinant DNA molecules (D. Hamer and P. Leder, personal communication) have shown that non-spliced viral transcripts do not accumulate in a stable form. As the undifferentiated cells seem to be unable to splice primary transcripts, this might explain the very low levels of virus-specific RNA in SV40-infected F-9 cultures.

The resistance of F-9 cells to viral infection is not limited to SV40. In addition, polyoma virus^{1,6} and ecotropic murine C-type viruses^{2,3} are not expressed in undifferentiated, embryonal carcinoma cells. Note that each of these viruses induces spliced mRNAs. A number of other viruses including Sindbis, encephalomyocarditis virus, and vaccinia virus² can grow in the undifferentiated embryonal carcinoma cells; but these viruses have not been shown to require splicing for their mRNA maturation. In contrast, the maturation of adenovirus 2 mRNA relies on a splicing mechanism; yet this virus is expressed at low levels in the undifferentiated cells⁵. We therefore suggest that adenovirus may use a distinct processing system in F-9 cells. Such enzymes could be cellular, virus induced or virus coded. The appropriate splicing of SV40 RNA only in the differentiated mouse cells⁴ raises the possibility that cell maturation itself is based on the expression of splicing enzymes. It is important to determine if only those mRNAs encoding differentiated functions require splicing, and if the enzymes required for the steps in RNA maturation such as splicing are induced in differentiating cells.

Using the method of Strickland and Mahdavi⁸ we have been able to induce the F-9 teratocarcinoma cell line to differentiate. The differentiated cells, after SV40 infection, contain spliced early SV40 mRNAs and synthesise SV40 T-antigen as demonstrated by immunofluorescence and immunoprecipitation (S.S. and G.K., in preparation).

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- Swartzendruber, D. E. & Lehman, J. M. *J. cell. Physiol.* **85**, 179 (1975).
- Teich, N. M., Weiss, R., Martin, G. R. & Lowy, D. R. *Cell* **12**, 973-982 (1977).
- Péries, J., Alves-Cardoso, E., Canivet, M., Debons-Guillemain, M. C. & Lasneret, J. *J. natn. Cancer Inst.* **59**, 463-465 (1977).
- Topp, W., Hall, J. D., Rifkin, D., Levine, A. J. & Pollack, R. *J. cell. Physiol.* **93**, 269-276 (1977).
- Kelly, F. & Boccardo, M. *Nature* **262**, 409-411 (1976).
- Swartzendruber, D. E., Friedrich, T. D. & Lehman, J. M. *J. cell. Physiol.* **93**, 25 (1977).
- Bernstine, E. G., Hooper, M. L., Grandchamp, S. & Ephrussi, B. *Proc. natn. Acad. Sci. U.S.A.* **70**, 3899-3903 (1973).
- Strickland, S. & Mahdavi, V. *Cell* **15**, 393-403 (1978).

9. Sherman, M. I. & Miller, R. A. *Dev. Biol.* **63**, 27–37 (1978).
10. Simmons, D. T., Takemoto, K. K. & Martin, M. A. *J. Virol.* **24**, 319–325 (1977).
11. Tegtmeyer, P., Rundell, K. & Collins, J. K. *J. Virol.* **21**, 647–657 (1977).
12. Prives, C. & Beck, Y. *Inserm* **59**, 175–187 (1977).
13. Crawford, L. V. *et al. Proc. natn. Acad. Sci. U.S.A.* **75**, 117–121 (1978).
14. Edidin, M., Patthey, H. L., McGuire, E. J. & Sheffield, W. D. in *Embryonic and Fetal Antigens in Cancer*, 239–248 (Oak Ridge National Laboratory, Tennessee, 1971).
15. Edidin, M., Gooding, L. R. & Johnson, M. *7th Symposium, Immunological Approaches to Fertility Control*, 1–19. (Karolinska Symposia on Research, 1974).
16. Kuff, E. L., Ferdinand, F.-J. & Khoury, G. *J. Virol.* **25**, 28–36 (1978).
17. Gariglio, P. & Mousset, S. *FEBS Lett.* **56**, 149–155 (1975).
18. Ferdinand, F.-J., Brown, M. & Khoury, G. *Virology* **78**, 150–161 (1977).
19. Khoury, G. & May, E. *J. Virol.* **23**, 167–176 (1977).
20. Khoury, G., Byrne, J. C., Takemoto, K. K. & Martin, M. A. *J. Virol.* **11**, 54–60 (1973).
21. Berk, A. J. & Sharp, P. A. *Cell* **12**, 721–732 (1977).
22. Berk, A. J. & Sharp, P. A. *Proc. natn. Acad. Sci. U.S.A.* **75**, 1274–1278 (1978).
23. Lai, C.-J., Dhar, R. & Khoury, G. *Cell* **14**, 971–982 (1978).
24. May, E., Kress, M. & May, P. *Nucleic Acids Res.* **9**, 3083–3099 (1978).
25. Lai, C.-J. & Khoury, G. *Proc. natn. Acad. Sci. U.S.A.* (in the press).
26. Gruss, P. *et al. Proc. natn. Acad. Sci. U.S.A.* (in the press).
27. Shih, T. Y., Khoury, G. & Martin, M. A. *Proc. natn. Acad. Sci. U.S.A.* **70**, 3506–3510 (1973).

The relationship of degradative and resistance plasmids of *Pseudomonas* belonging to the same incompatibility group

THE fluorescent pseudomonads include *Pseudomonas* which can cause serious infections in man, and soil bacteria such as *P. putida*. Resistance (R) plasmids have been found in strains of both species and degradative (D) plasmids have been demonstrated in strains of the latter species. Where such plasmids can be transferred by conjugation to a common host strain, it has been possible to establish whether they can stably co-exist. Inability to co-exist (incompatibility) is the basis of their classification^{1,2}. It has recently been suggested that both the IncP-2 and IncP-9 groups contain both R and D plasmids^{2,3}. In addition the IncP-2-plasmids are both very large⁴. Among R plasmids of enterobacteria, incompatibility is generally an indication of evolutionary relationship in that the DNAs of incompatible plasmids show greater homology with each other than with those of compatible plasmids⁵. However, cases of incompatible plasmids lacking significant homology have also been reported^{5,6}. We have examined the extent to which two R plasmids from *P. aeruginosa* and two D plasmids, one from *P. putida* and the other from *P. aeruginosa* (all assigned to the IncP-9 group^{3,15,16}) share common sequences. The results suggest that plasmids of the same incompatibility group are not necessarily closely related.

The plasmids used in this study are described in Table 1. Plasmid pMG18 carries the same resistance determinants as R2, plus several additional ones. Plasmids NAH and TOL encode

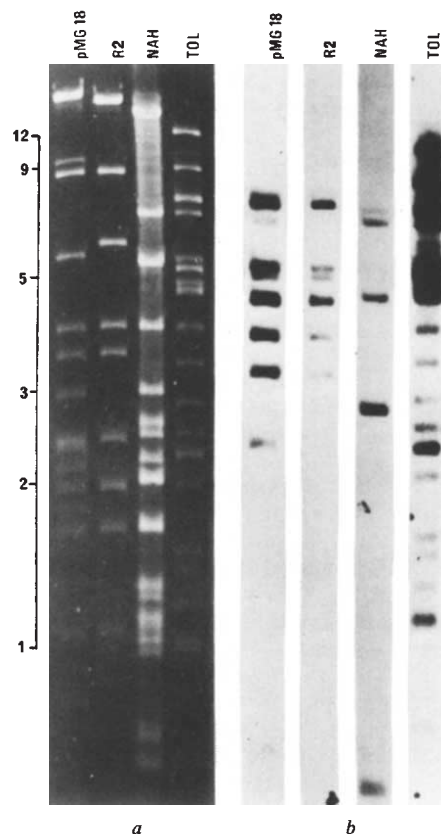


Fig. 1 *a* Banding patterns of *Eco*RI-digested plasmid DNA after electrophoresis on 1% agarose gels and staining with ethidium bromide. *b* Autoradiographs obtained by transferring *Eco*RI-digested fragments of TOL DNA to nitrocellulose filters by the Southern blot technique, and hybridising with the four different ³²P-labelled plasmid probes¹⁰. Probe DNA was labelled by 'nick translation' with [³²P]-dCTP and sheared by sonication to an average length of 500 base-pairs. Hybridisation was performed in Denhardt's solution²⁰ at 65 °C for 18 h. Autoradiography was carried out for 24 h and then for a further 3 days to reveal weakly hybridising bands. The autoradiographs presented here were exposed for 24 h.

the degradation of naphthalene and toluene respectively. In the course of both degradations, catechol is cleaved and degraded via the *meta* (α -ketoacid) pathway. However, the R plasmids have no known function in common with the D plasmids.

Overall homology was established by DNA:DNA hybridisation on nitrocellulose filters¹⁷. Plasmid pMG18 contained sequences that were homologous with most, if not all, of R2 (Table 2). The extent of homology in the reciprocal hybridisation (67%) was close to that expected (69%) from this experiment, given that pMG18 is larger than R2 (100 kilobases compared with 73 kilobases).

TOL DNA showed significant homology with both R plasmids (Table 2). Given that the sizes of TOL and R2 are 117 and 73 kilobases respectively, the TOL:R2 hybridisation showed that the two plasmids shared sequences of DNA equivalent to about 11 kilobases (the average of 117×0.11 and 73×0.14). Similarly, we calculated that the DNA sequences common to TOL and pMG18 are equivalent to 20 kilobases (117×0.17 and 100×0.20). However, the R plasmids shared much less homology with NAH; here, the common sequences were only equivalent to about 3 kilobases.

Previous data had suggested that NAH and TOL were related¹⁰. We found that the extent of this relationship was small. The degree of reassociation of TOL DNA probe with unlabelled NAH DNA was $12 \pm 2\%$, and the reciprocal hybridisation gave $8 \pm 3\%$ reassociation. Thus, we calculated the sequences common to both plasmids as being equivalent to only 9 kilobases (72×0.12 and 117×0.08).

Table 1 Plasmids used in this study

Plasmid	Original host strain	Host strain in this work	Phenotype	Approximate size of plasmid DNA (kilobase-pairs) [†]	Ref.
R2	<i>P. aeruginosa</i>	PU21	CbSmSu	73	7
pMG18	<i>P. putida</i>	PU21	CbGmKmSmSuHg	100	8
NAH	<i>P. putida</i>	PpG7	Nah	72	9, 10
TOL	<i>P. putida</i>	PaW1	Tol	117	11

Abbreviations: Cb, Gm, Km, Sm, Su, Hg: resistance respectively to carbencillin, gentamicin, kanamycin, streptomycin, sulphonamide and Hg²⁺. Nah, Tol: ability to use naphthalene and ability to utilise toluene and *m*- and *p*-xylenes.

* R2 and pMG18 were transferred from *P. aeruginosa* strain PU21¹², which carries cryptic plasmids¹³, to a plasmid-free *P. putida* strain, AC34¹⁴.

† The plasmid sizes were estimated from the sum of the sizes of the fragments derived from *Eco*RI cleavage and resolved on agarose gels. Fragments of λ DNA run on the same gels were used as reference markers.

To localise the related segments of the R and D plasmids and to confirm that the observed homology between NAH and the R plasmids was significant, a series of hybridisations was performed, using radioactively-labelled probe DNAs of the plasmids and Southern blots of endonuclease-generated fragments of unlabelled plasmid DNAs¹⁰.

The close similarity in size between most of the R2 fragments and a series of pMG18 fragments again suggested that R2 was very closely related to pMG18 (Fig. 1a). This was borne out by autoradiography, which showed that all R2 bands hybridised with pMG18 probe, and all but three bands in the pMG18 pattern showed homology with R2 probe (data not shown).

In the filter hybridisations described above, pMG18 showed more homology with TOL than did R2. As expected from this result, TOL probe hybridised with more pMG18 bands than R2 (data not shown). These findings were confirmed in reciprocal experiments with pMG18 and R2 probes, which hybridised with the same resolved bands of TOL DNA, plus, in the case of pMG18, several additional bands (Fig. 1b). Since these represented small fragments, they were insufficient to account for the difference between 20 and 11 kilobases. However, some of the TOL bands which showed homology with both R plasmid DNAs were composed of more than one fragment, and comparison of the autoradiographs suggested that pMG18 probe was hybridising to a greater degree to some of those bands than did R2 probe.

The results of the NAH hybridisation against TOL DNA essentially confirm those of Heinaru *et al.*¹⁰ for the SAL plasmid, which is very closely related to NAH: that is, homology was largely confirmed to a few bands (Fig. 1b). Of these, band 4 in the TOL fragmentation pattern, composed of one fragment species²¹, is lost from the Tol⁻ excision derivative of TOL, pWVO-8 (ref. 22). Only one TOL band showed some homology with all the probes. However, since this band was made up of more than one fragment species, we cannot say if the same fragment hybridised in each case: indeed, the homology could also be in different portions of one fragment.

When each of the R plasmid DNAs was used to probe the NAH digestion pattern, they only hybridised (relatively weakly) with the 5.8-kilobase band. This band was also one of those which hybridised with TOL probe. These findings suggest the existence of a very small 'core sequence' common to all four plasmids. Therefore, for these four plasmids of the IncP-9 group, incompatibility cannot be taken to imply more than a very limited amount of evolutionary relationship.

Table 2 Homology among R and D plasmids of *Pseudomonas* belonging to the IncP-9 incompatibility group

Total unlabelled DNA from strain-carrying plasmid	Percentage of reassociation with labelled plasmid DNA from:			
	TOL	NAH	R2	pMG18
TOL	100±6	12±2	14±3	20±2
NAH	8±3	100±2	5±1	2±1
R2	11±3	4±1	100±3	67±2
pMG18	17±4	6±2	98±3	100±2

Plasmid DNA was isolated by SDS lysis followed by NaCl-SDS precipitation of chromosomal DNA¹⁸ and purified by CsCl-ethidium bromide density ultracentrifugation; it was then labelled *in vitro* with [α -³²P]dCTP by 'nick translation' (ref. 19), and sheared by sonication to an average size, as determined by agarose gel electrophoresis, of 500 base pairs. The DNA was heat-denatured and used to probe an excess of unlabelled single-stranded whole cell DNA from the same plasmid-carrying strains bound to nitrocellulose filters. The hybridisation was performed at 37°C for 18 h in 2×SSC/50% (w/v) formamide under paraffin oil¹⁷. The values shown are the percentages of reassociation ($\pm$ s.d.) of ³²P-labelled plasmid DNA with unlabelled plasmid DNA. Data are of the mean of eight separate determinations, after deduction of the values from control experiments using labelled probe and DNA from the plasmid-free host strains. These control values were about 5% of those for hybridisations with DNA from cells carrying the homologous plasmid.

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1. Sagai, H. *et al.* *Antimicrob. Agents Chemother.* **10**, 573-578 (1976).
2. Jacoby, G. A. in *Microbiology—1977* (ed. Schlessinger, D.) 119-126 (American Society for Microbiology, Washington, 1977).
3. Korffhagen, T. R., Sutton, L. & Jacoby, G. A. in *Microbiology—1978* (ed. Schlessinger, D.) 221-224 (American Society for Microbiology, Washington, 1978).
4. Hansen, J. B. & Olsen, R. H. *Nature* **274**, 715-717 (1978).
5. Grindley, N. D. F., Humphreys, G. O. & Anderson, E. S. *J. Bact.* **115**, 387-398 (1973).
6. Willshaw, G. A., Smith, H. R. & Anderson, E. S. *Molec. gen. Genet.* **159**, 111-116 (1978).
7. Kawakami, Y., Mikoshiba, F., Nagasaki, S., Matsumoto, H. & Tazaki, T. *J. Antibiot., Tokyo* **25**, 607-609 (1972).
8. Summers, A. O. & Jacoby, G. A. *Antimicrob. Agents Chemother.* **13**, 637-640 (1978).
9. Dunn, N. W. & Gunsalus, I. C. *J. Bact.* **114**, 974-979 (1973).
10. Heinaru, A. L., Duggleby, C. J. & Broda, P. *Molec. gen. Genet.* **160**, 347-351 (1978).
11. Williams, P. A. & Murray, K. *J. Bact.* **120**, 416-423 (1974).
12. Jacoby, G. A. *Antimicrob. Agents Chemother.* **6**, 239-252 (1974).
13. Pemberton, J. M. & Clark, A. J. *J. Bact.* **114**, 424-433 (1973).
14. Palchoudhuri, S. & Chakrabarty, A. *J. Bact.* **126**, 410-416 (1976).
15. Austen, R. A. & Dunn, N. W. *Aust. J. Biol. Sci.* **30**, 357-366 (1977).
16. Jacoby, G. A. & Matthew, M. *Plasmid* **2**, 41-47 (1979).
17. Roussel, A. F. & Chabbert, Y. A. *J. gen. Microbiol.* **104**, 269-276 (1978).
18. Guerry, P., LeBlanc, D. J. & Falkow, S. *J. Bact.* **116**, 1064-1066 (1973).
19. Maniatis, T., Jeffrey, A. & Kleid, D. G. *Proc. natn. Acad. Sci. U.S.A.* **72**, 1184-1188 (1975).
20. Denhardt, D. T. *Biochem. Biophys. Res. Commun.* **23**, 641-646 (1966).
21. Duggleby, C. J., Bayley, S. A., Worsley, M. J., Williams, P. A. & Broda, P. *J. Bact.* **130**, 1274-1280 (1977).
22. Bayley, S. A. *et al.* *Molec. gen. Genet.* **154**, 203-204 (1977).

Electron microscopic evidence for the circular form of RNA in the cytoplasm of eukaryotic cells

LINEAR single-stranded RNA genomes extracted from several RNA viruses were found to exist in a circular conformation when examined in the electron microscope in partially denaturing conditions¹⁻⁴. The circular conformation of viral RNA presumably arises from base pairing between RNA sequences at the two ends of the RNA molecules. The biological significance of the circular structure of single-stranded RNA is still unknown. Here we report the observation of the circular form of RNA extracted from the cytoplasm of several eukaryotic cells.

Cytoplasmic RNA was extracted from HeLa cells by the procedure of Spradling *et al.*⁵. RNA were dissolved in buffer containing 0.05-0.1 M Tris, 0.005-0.001 M EDTA, pH 8.5, and 40-92% formamide, and spread using the basic protein monolayer technique⁶. When examined in the electron microscope, about 1-2% of the molecules could be seen to be in circular form even in highly denaturing conditions (92% formamide, 0.05 M Tris, 0.005 M EDTA, pH 8.5). The electron micrographs of some of the circular molecules observed are shown in Fig. 1. RNase treatment completely eliminated these molecules, suggesting that they are RNA molecules. As control, influenza virus RNA and HeLa cell ribosomal RNA were examined. No circular RNA molecules were observed in the electron microscope in the above conditions. 23S and 16S ribosomal RNA extracted from *Escherichia coli* also do not show any circular structure¹. Therefore, we consider it unlikely that the circular structure observed in HeLa cell cytoplasmic RNA is due to random cross-over of the two ends of the RNA molecules. Furthermore, circular forms were observed even when the molecules were highly stretched by the spreading force.

'Pan-handles' were usually observed in the circular RNA. One of these presumably represents the RNA-RNA duplex formed by the base pairing of the 3'- and 5'-ends of an RNA molecule. Sometimes, the pan-handles exist in the form of 'rabbit ears' (see Fig. 1). That these structures could still be

observed in a highly denaturing condition suggested that these structures have a high degree of sequence homology and a high G-C content. Alternatively, these circular RNAs could be covalently closed as in viroid RNA⁷. The possibility that the circular RNA molecules are due to protein linkage can be ruled out because proteinase K treatment, which we did not use routinely although it is part of the RNA extraction technique⁵, did not reduce the number of circles. In circular RNA molecules containing two pan-handles the pan-handles are often at an equal distance from each other.

The length distribution of circular HeLa cell cytoplasmic RNA is shown in Fig. 2. It is interesting that the size distribution seems to be of a multiple integral of 300 nucleotides ($\sim 0.22 \mu\text{m}$). However, 300 nucleotide-long circles were not observed, probably as they are too small to be seen in the electron microscope.

The circular forms of RNA were also observed in the poly(A)⁺ fraction of HeLa cell cytoplasmic RNA, although the length distribution is skewed to the shorter range (Fig. 3). Circular RNA can also be seen in RNA extracted from HeLa cell nuclei. However, the amount of circular RNA observed was greatly reduced when the nuclei were washed with detergent and extracted as described by Penman⁸. These results suggest that the majority of circular RNA is probably localised in the cytoplasm and not in the nucleus. Cytoplasmic RNA isolated from CV-1 cells (a monkey kidney cell line) and Chinese hamster ovary cells, and total RNA isolated from *Physarum polycephalum*, were also found to contain similar circular RNA molecules.

The biological significance of circular RNA is unknown. In the case of viral RNA it has been suggested that the pan-handles at the ends of RNA molecules may serve as the initiation site for

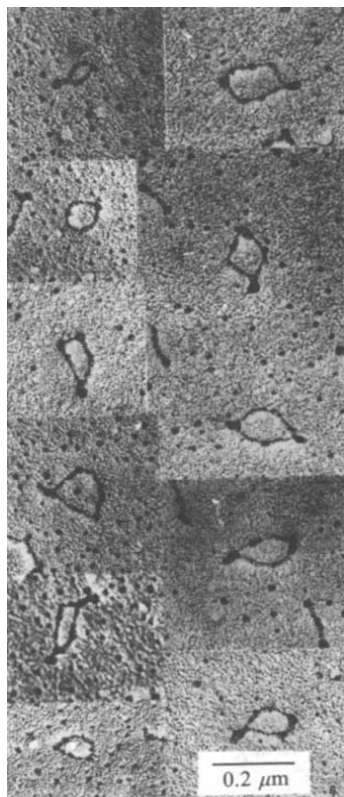


Fig. 1 Electron micrographs of circular RNA molecules extracted from the cytoplasm of HeLa cells. HeLa cell cytoplasmic RNA extracted according to Spradling *et al.*⁵ was precipitated with ethanol at -20°C and resuspended in 92% formamide, 0.05 M Tris, 0.005 M EDTA, pH 8.5, $50 \mu\text{g ml}^{-1}$ cytochrome c and spread on hypophase containing 50% formamide, 5 mM Tris and 0.5 mM EDTA, pH 8.5. The RNA molecules were picked up on a palladium-coated grid and processed for electron microscopy as described elsewhere¹⁰.

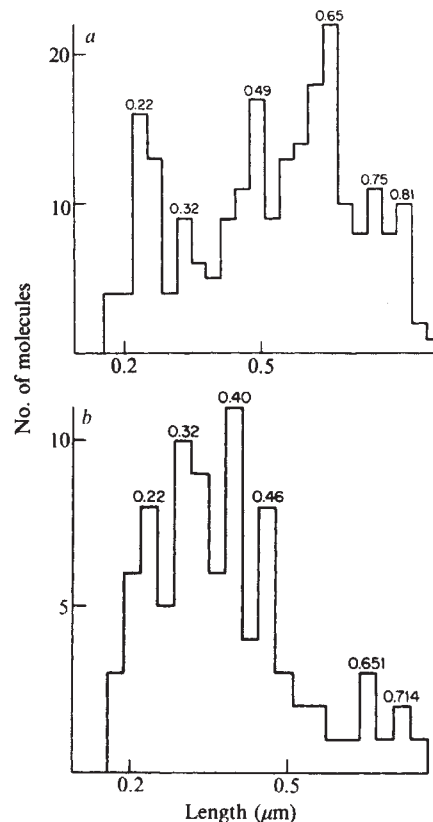


Fig. 2 Histogram of length of HeLa cell total cytoplasmic circular molecules (a) and cytoplasmic poly(A)⁺ circular RNA molecules (b).

viral RNA replication^{1,2}. The circular RNA that we observed in the cytoplasm may represent cryptic viral RNA or viral RNA in chronic infection. If such were the case, one may be able to detect RNA replication activity in the post-mitochondrial cytoplasmic fraction. On the other hand, circular RNA may represent efficient templates for translation in which the ribosome running off the mRNA template at the 3'-end immediately re-associates with mRNA at the 5'-end to start new initiation of protein synthesis. This model was suggested by Baglioni *et al.*⁹ to account for the slow rate of exchange of ribosomal subunits and the stability of mRNA in mammalian cells. Such a model would predict the close association of the cap structure at the 5'-end and the poly(A) sequence at the 3'-end of mRNA molecules. Partial nuclease digestion of mRNA molecules would result in the enrichment of the cap structure in RNA fragments containing the poly(A) sequence. These possibilities require experimental verification.

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- Hsu, M. T., Kung, H. J. & Davidson, N. *Cold Spring Harb. Symp. quant. Biol.* **39**, 943-950 (1973).
- Hewlett, M. M., Pettersson, R. F. & Baltimore, D. *J. Virol.* **21**, 1085-1093 (1977).
- Kolářofsky, D. *Cell* **8**, 547-555 (1976).
- Samson, A., Boyloy, M. & Hannoun, C. *C. heb. Seanc. Acad. Sci., Paris D282*, 1653-1655 (1976).
- Spradling, A., Hui, H. & Penman, D. *Cell* **4**, 131-137 (1975).
- Davis, R. W., Simon, M. & Davidson, N. *Meth. Enzym.* **21**, 414-428 (1971).
- Gross, H. J. *et al. Nature* **273**, 203-206 (1978).
- Penman, S. *J. molec. Biol.* **17**, 117-130 (1966).
- Baglioni, C., Vesco, C. & Jacobs-Lovena, M. *Cold Spring Harb. Symp. quant. Biol.* **34**, 555-566 (1969).
- Hsu, M.-T. *J. Bact.* **118**, 425-428 (1974).

reviews

Worthwhile science

J. R. Ravetz

Science and Its Critics. By John Passmore. Pp. 102. (Duckworth: London, 1978.) Paperback £2.95.

AN advocate of an unpopular cause could not wish for an interpreter more judicious and sympathetic than John Passmore. Continuing his series of surveys of the deeper problems of our scientific world-view, in *Science and Its Critics* he handles some very controversial matters with unfailing erudition and tact.

Through four themes, he reviews the great variety of criticisms that have been made of science. The easiest to handle is the first, the claim that scientific explanation is defective as a means of real knowing. The many ambiguities in the concept of "explanation" are sufficient for the fragmentation of any line of criticism. The criticism of the destructive potential of science-based technology merits more consideration. Passmore refuses to allow the shelter of ignorance of consequences claimed by "pure" scientists. For our science is too bound up with technology, both in its present practice and in its basic philosophy as announced by Bacon and Descartes. Although the problem of personal responsibility of the scientist is not easily resolved either way, the collective involvement of science in its applications cannot legitimately be denied.

When he comes to "the scientific spirit", Passmore seems at last to be speaking more from knowledge of man than of books. He coins the useful term "aristoscience" relating to the elite practitioners of the elite fields. For that, the "spirit" requiring analysis is *hubris*. He described very compellingly how this affects the style of scientific work, right down to the self-serving criteria of choice of problems, the setting of inappropriate standards for "immature" sciences, and indeed an indifference to the welfare of ordinary people provided that scientists are well fed.

Finally, he talks of "uniqueness, imagination and objectivity", still rebutting metaphysical attacks but admitting the strength of social criticisms. And this seems to be the verdict, as it emerges towards the end. As a social institution composed of people, science is not immune from any weakness or failing. But as an ideal for knowledge, placing man in a particular relationship to the Universe, science, as the successor to the philosophies of the Renaissance and the

Enlightenment, expresses the essential values of our civilisation.

Thus, in a bare hundred pages Passmore has introduced the whole field of criticism of science, including quotes from all the significant authors. Its only defect as an introductory text for students is that the argument flows rather too smoothly from one topic to the next; another edition with sections and break-headlines would much enhance its pedagogical value.

This book was written in response to the crisis of the 1960s; John Passmore might now begin working on the sequel which will be necessary for the 1980s. This epoch has just begun, inaugurated by the nuclear accident at the Harrisburg plant,

Three Mile Island, Pennsylvania. In America the Press carried reflective stories with such headlines as "The Nuclear Experts Can't Be Trusted" and "Credibility Crap": "One of the first casualties of the Three Mile Island nuclear accident was scientific credibility..." (*Newsweek*, 23 April, 1979). How "scientists" could be involved in the same processes of prevarication, lies and cover-up as the Vietnam or Watergate politicians, is a problem which, I believe, must be considered urgently by those who would understand and defend what is worthwhile in science in the coming period. □

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Hückel theory

Hückel Theory for Organic Chemists. By C. A. Coulson, B. O'Leary and R. B. Mallion. Pp. 182. (Academic: London, New York and San Francisco, 1978.) £9.50; \$19.65. *Hückel Molecular Orbital Theory.* By K. Yates. Pp. 371. (Academic: New York, San Francisco and London, 1978.) £20.80; \$32.

ORGANIC chemists are beginning to appreciate that an elementary knowledge of molecular orbital theory in its simplest form — Hückel theory — helps greatly in understanding the properties and reactions of organic molecules. These books are not the first to attempt the exposition of Hückel theory for the organic chemist, and they will not be the last; but it is possible that one of them will turn out to be the best and the other the worst.

Charles Coulson was loved by all who knew him, and deeply respected both as a scientist and as a teacher. Hückel's name is given to the theory, but it was Coulson who, first with Rushbrooke and later with Longuet-Higgins, developed it and showed how powerful it could be, for all its simplicity. He lectured on it to organic chemists in Oxford, and in 1971 the lectures were recorded on magnetic tape. Mallion and O'Leary have now transcribed the recorded lectures into print, and although they have been unable to resist the temptation to add some pretty mathematical results of their own, they have confined themselves to appendices and footnotes. The rest is Coulson: precise but not pedantic, simply expressed but not

condescending and always honest and good-humoured. One can quibble about a few matters (is the ω technique of any interest now? Is free valence of any use to anybody?) but this is as thorough and readable an account of the principles of Hückel theory as we are likely to see. There are a few misprints, and a few other irritations, such as the use of "less-approximate" to mean "more accurate" and the pervasive hyphenation of adjectival phrases, as in "reasonable (but-not-exactly-correct) supposition", but these are unimportant. The prospective purchaser may be further encouraged by the knowledge that the royalties will go to the Coulson Memorial Fund at Oxford, to support foreign students of theoretical chemistry.

It must be said, however, that this excellent book has limitations in its coverage, and these limitations will be of particular concern to the very people who are expected to read it. The applications of Hückel theory in predicting reactivity are described only briefly, and the more modern ideas which have flourished in the wake of the 'Woodward-Hoffman rules' are not mentioned at all. The book provides an excellent introduction to the basic molecular orbital theory, but it does not completely serve the needs of the modern organic chemist. It is therefore not surprising that the same publisher is responsible for the book by Yates, which seeks to cover much more thoroughly these important new ideas. It is unfortunate, perhaps, that the books arrived for review almost simultaneously, for the virtues of the one emphasise the deficiencies of the other. An example of the difference is given

by the treatment of the Born–Oppenheimer approximation, which is implicit in the Hückel method but need not be made explicit in an elementary treatment. Coulson accordingly does not mention it at all. Yates mentions it twice — once to say that it must be used, and once to say that it has been used. He does not say what it is. Another example is the treatment of the Coulson–Rushbrooke pairing theorem, which is fundamental to many of the applications of Hückel theory, and important in understanding the unexpectedly wide validity of the theory. Coulson naturally proves the theorem, carefully and precisely, so that there is no doubt about the exact assumptions which are required. Yates has no reference by name to the theorem, either in the index or, as far as I can discover, in the text; and although he could have stated the essence of the theorem, as Coulson does, in a dozen lines, he spreads the statement over several pages, calls it a series of generalisations rather than a theorem, and moreover, by introducing a further weaker generalisation, for which he gives an invalidating counter-example, leaves the reader with the impression that all of these ‘generalisations’ share the same lack of generality.

Yates claims, with some justice, that organic chemistry students frequently apply important ideas and approaches such as the Woodward–Hoffman rules “without a sufficiently sound understanding of their theoretical basis”. Yet he fobs his readers off with phrases like “it can be shown that . . .” or “it turns out that . . .”, even where the result could be derived quite readily. Other arguments are presented in a thoroughly superficial manner. For example, the important class of sigmatropic reactions is dealt with on the basis that “the important molecular orbital of the π framework involved in the migration is considered by Woodward and Hoffman to be the [highest occupied one]”. Is the student to take this *ex cathedra* statement as the basis of his sound understanding? There is no discussion of the reasons for the validity of this assumption; nor is there any cross-reference, or index reference, to a later chapter where this class of reaction is studied by another method.

Indeed, there is an irritating lack of cross-references throughout: the reader is continually being told that a certain topic will be treated “later”, with no cross-reference to lead him to the right place. The index too, is thoroughly unhelpful: for example, of the four entries under “Woodward–Hoffman rules” one leads the reader to the statement that they will be treated “in the following chapters”, and another to the statement that they will be described “later”. None of them directs the reader to the full derivation of the rules or even to a statement of what they are.

This lack of consideration for the reader is illustrated in a different way by a sentence in the first chapter, which reads:

“For the second molecular orbital . . . a similar treatment would give $E_2 = (\alpha - \beta) / (1 - S)$, and since β is generally greater in absolute magnitude than α (and both are negative energy terms) this corresponds to a positive energy level or an antibonding molecular orbital”. Now the author has slipped up in his statement (repeated in a diagram) that antibonding orbitals have positive energy. They merely need to have energy greater than α . If the energy had to be positive, and α and β were both negative, then it would certainly be necessary that $|\beta| \gg |\alpha|$. But this is not the case; for with the normal choice of energy scale (adopted implicitly if obscurely on the previous page) α is typically around -15 eV and β around -3 eV. Any attempt to check the relative values of $|\beta|$ and $|\alpha|$ would have revealed this fact, and, eventually, the error about antibonding energies; but the author seems to have been content to state what “must”

be true. He has advertised his uncertainty by adding the word “generally”, but evidently without contemplating the consequences of $|\beta|$ being less than $|\alpha|$. He also seems to be unaware that it is meaningless in any case to compare values of α and β without specifying the energy datum precisely, as they change in different ways when the datum is changed, and indeed either can be made to vanish by a suitable choice.

Let us then turn back to Coulson, O’Leary and Mallion. They will not take the reader through the important modern ideas in theoretical organic chemistry, but they will provide him with a secure foundation on which to base his study of the more specialised monographs in the field.

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Nature-nurture

Intelligence: Heredity and Environment. By P. E. Vernon. Pp. 390. (Freeman: San Francisco and Reading, 1979.) Hardback £10; paperback £5.30.

VERNON sets out to reconcile the conflicting claims regarding intelligence and its transmission and to adopt an eclectic position that gives due weight to all the relevant facts. To this end he presents a critical appraisal of a very large number of studies with admirable lucidity.

Although such eclecticism may sound impersonal, there certainly is no lack of personal involvement on Vernon’s part, and it is this as much as anything which gives the book its fascination. In many ways it is an intensely personal statement, from the preface in which he speaks of this book as his last, to the finale in which he mounts an emotive attack on recent trends in childrearing and education and contemplates the breakdown of Western mores and standards.

As the author looks back on the half century during which he himself has made such a distinguished contribution to psychometrics, he clearly perceives that all is not well. To take a specific example, intelligence testing is being banned in some parts of the United States on grounds of cultural bias. Vernon devotes a chapter to effectively countering this accusation. But more generally, psychometrists seem to be an increasingly isolated group, disowned on the one hand by many geneticists and on the other by many developmental psychologists. From the beginning quantification has been the primary goal in psychometrics, and has arguably hindered rather than helped conceptualisation. Vernon argues that “psychometry is quite entitled to use its own brand of operationalism,

regardless of philosophical theories of scientific method, provided it works”. But does it work?

The bulk of the book is devoted, as the title suggests, to the nature-nurture issue. Vernon makes a plea for scientific objectivity, suggesting that questions of social and political reform are not the business of the psychologist; it is his business simply to supply scientific data. In the context of his argument it is particularly unfortunate that Vernon has been forced to acknowledge (in a prefatory notice) the evidence of systematic fraud having been perpetrated by Burt. Vernon’s citations of Burt are exceeded only by those of Jensen, and even given the author’s attempts to minimise his dependence on Burt’s studies the affair hardly encourages us to accept the impartiality of scientific data.

The book gives detailed coverage of the heritability studies and the criticisms which have been raised against them. Although Vernon offers a stalwart defense of the attempt to estimate heritability, the sheer variety of methodological and statistical doubts raised by critics of the various studies may tend further to undermine the reader’s confidence.

Vernon’s equivocates on racial differences in intelligence, suggesting that it is highly probable that some genetic differences are involved, but that owing to the confounding of race with environmental differences it does not seem possible to separate their effects. To paraphrase the ancient Zen argument, it may be that the real problem lies not so much in our question being unanswerable, as in our remaining in the state of mind that led us to ask it.

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Advances in metabolism

The Year in Metabolism, 1977. Edited by N. Freinkel. Pp. 448. (Plenum Medical: New York and London, 1978.)

THIS volume is the second in what is proposed to be an annual series, the purpose of which is to review advances in metabolism. The publishers have assembled an impressive list of internationally recognised experts to produce twelve chapters on diverse topics. The appeal of a series like this depends not only on the appropriate choice of subjects; in order to attract the non-specialist, recent advances have to be made intelligible to those without extensive background knowledge. In both respects this volume is largely successful. If there is any unevenness of presentation it is because some of the authors have interpreted their brief literally and dealt only with recent advances, whereas others have written general reviews with extensive references to the earlier literature.

The review by Murad and Aurbach on cyclic GMP metabolism, particularly in relation to disease states, is timely and draws together data which is not readily to be found elsewhere. The chapter by Fajans on diabetes mellitus provides a useful account of genetic aspects and the linking of susceptibility with the HLA system and a possible viral aetiology. Insulin resistance is also discussed together with various aspects of treatment, and there is a good account of possible pathogenic mechanisms in the development of vascular complications.

Another chapter contains an authoritative account of glucagon and somatostatin by Unger. Topics discussed include structure-activity relationships, control of secretion and the bi-hormonal (glucagon and insulin) hypothesis of diabetes mellitus. Inevitably there is some overlap with the chapter by Felig and Koivisto on body fuel metabolism, in which the inter-relationships of glucagon, insulin and somatostatin are considered. Hirsch writes about obesity, discusses what is new and concludes not much. His philosophical discussion of causation and treatment ends on a pessimistic note for the sufferer. Lipids and lipoproteins are discussed by Goodman who selects highlights from a complex subject with care.

Rosenberg's chapter on amino acid disorders also selects a few topics for discussion, including phenylketonuria, the glutamyl cycle and oxoprolinuria. He also gives a fascinating account of the metabolic mechanisms underlying Jamaican vomiting sickness, which is due to the presence of the unusual amino acid, hypoglycin, in unripe ackee fruit.

Seegmiller offers an extensive and detailed account of how human disorders of purine and pyrimidine metabolism are linked to an immunodeficiency syndrome. Possible mechanisms are discussed.

The topic of divalent ion metabolism is covered expertly by Coburn, Hartenbower and Kleeman, who draw attention to how recent advances in vitamin D metabolism are enabling some of the clinical disorders to be better understood. The complex inter-relationships between PTH, vitamin D and calcium, phosphate and magnesium metabolism are summarised. Winick deals with the topic of nutrition, growth and development with particular emphasis on malnutrition, genetic obesity and nutrition in pregnancy. Finally, Williams provides a short but useful review of renal stone disease, and Lieber reviews the metabolism and metabolic actions of ethanol.

The same group of authors have been recruited to contribute to future volumes and this may introduce some difficulties in producing a wide enough variation in subject matter in future editions. It will be interesting to see whether the publishers and authors can maintain the same high standard to be found in this volume.

The book should appeal to a wide audience which should include biochemists concerned with human disease and teaching medical students, as well as endocrinologists and physicians with interests in metabolic disorders.

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Disorder in crystals

Disorder in Crystals. By N. G. Parsonage and L. A. K. Staveley. Pp. 926. (Oxford University Press/Clarendon: Oxford, 1979.) £28.

IN the hierarchy of matter — gas, liquid, crystal — crystals signify order, pattern; they represent classical discipline as against romantic anarchy. "Damn braces, bless relaxes!" cried that arch-romantic, Blake. The authors of this impressive text might have taken Blake as their mentor; their crystals are for ever relaxing from a state of Roman order into one of Italian disarray; same place, changed ways.

The book is about the various ways in which crystals can lose perfect regularity without ceasing to be crystals: it is a study of local variability superimposed on a basic periodicity. The authors in their Introduction (which neatly summarises the argument of the book) distinguish three types of disorder: disorder of position, orientational disorder and magnetic disorder. These might respectively be exemplified by Cu₃Au, where copper and gold atoms are apt to trespass on each other's appointed lattice sites; NH₄Cl, in which a triad axis of the (NH₄)⁺ ion can point in different defined directions and the ion itself can execute a click-stop rotation about a triad; and CsCoCl₃·2H₂O, containing one-dimensionally ordered antiferromagnetically coupled chains of tilted 'Ising spins'. Any chemist, physicist or metallurgist, however expert he may be in one or other of these very broad categories of disorder, will discover a great variety of parallels to and variants of the particular kind of behaviour he is familiar with. The book is a remarkable achievement of synthesis.

The text begins with a detailed treatment

of the thermodynamic and statistical mechanical approaches to phase transformations and degrees of order: it is a mark of the combined rigour and flexibility of the authors that they do not agonise about the propriety of classifying order/disorder transformations as phase transformations. The Ising lattice theory in various dimensions receives a full historical survey, as do the various thermodynamic approaches to the order of transformation; the concept of the λ transformation is frequently cited throughout the book. Even such a technical subtlety as the bond percolation problem (if water be imagined capable of percolating along one type of bond only in a crystal, what proportion of the atoms would be wetted if the crystal were bathed in water?) gets due attention.

There is next a useful chapter on experimental techniques, such as thermochemical measurements, diffraction, NMR, IR and Raman spectroscopy, dielectric permittivity, dispersion and loss measurements, and others. The aim is to explain what kinds of information the various methods can provide, and why; no attempts are made to describe hardware or practical difficulties.

The authors then settle down to particular materials, starting with positional order in alloys. As a metallurgist, the reviewer can confirm that this chapter is reasonably up to date and quite clear in classifying and exemplifying the types of positional order. No attention is devoted here, and little elsewhere in the book, to the kinetics of order-disorder transformations, which is a major topic of current metallurgical concern; and microstructure — for instance, the curious structures resulting from spinodal decomposition — is not considered. Again, some important concepts are not explained but simply used: the notion of 'antiphase domain' is an example. This omission of

definitions happens at intervals in both the theoretical and experimental parts of the book but, given its ambitious scope, this could hardly have been avoided.

The next five chapters (432 pages) constitute the heart of the book. The first, dealing with positional disorder in inorganic compounds, covers in particular the currently important topic of superionic conductors such as an allotrope of AgI, and beta-alumina. (The authors say of AgI that "this singular crystal has often been described as consisting of fluid silver ions in a solid lattice of iodide ions, and even as a 'missing link' between the solid and liquid states".)

A long and illuminating chapter follows on orientational disorder in salts, pride of place going to ammonium salts and including a treatment of ferroelectric salts. This is followed by a clear dissection of the exceedingly involved polymorphs of ice, most of them created by high pressure, and the various descendants of Pauling's statistical mechanics of hydrogen bonds in ice are described.

Two chapters follow on molecular solids, with special emphasis on compounds (such as perfluorocyclohexane) which contain both fixed and labile constituents. Such compounds, called 'rotator phases' or 'plastic crystals', in which some ions exhibit extreme orientational disorder, have remarkable properties such as extreme softness and very rapid diffusion, at appropriate temperatures. Hydrogen and deuterium, various linear and non-linear molecules, and many other groups are analysed, and the chapter finishes with a valiant attempt to systematise the statics and dynamics of these variegated molecular groupings. The concept of a glassy crystal (in which disorder of ions on a sublattice is frozen in on cooling) is introduced. Long-chain monomer molecules are treated quite fully, but degrees of order in polymers are not discussed at all. A special feature in these chapters is the close attention paid throughout to the implications of thermal analysis and, in particular, of the entropy changes associated with order transformations.

A further chapter discusses clathrates (compounds such as β -quinol with large near-spherical structural voids that can be partially filled with 'guest compounds', which behave as independent non-interacting parasites on the host), channel compounds of urea and thiourea, and the metallic intercalates of graphite studied in recent years. An example of what can be deduced from entropy measurements is the authors' conjecture that cyclooctane molecules caught in thiourea channels undergo conformational changes, limited by the channel geometry, at sharply defined temperatures. It would be intriguing to know how polymer chains would behave if constrained within such a host.

The final chapter deals with inorganic

magnetic compounds, first in quantum-mechanical generality and then in taxonomic and specific detail. The reader is taken through the luxuriance of phenomena such as ferromagnetism, antiferromagnetism, spin waves, metamagnetism, helical spins, and their hybrids.

After this plethora, it is only proper to outline what the authors have omitted. As already mentioned, polymers are excluded. There is no reference to liquid crystals, that other missing link, which is a pity since, in terms of statistical mechanics, they show a temperature dependence closely similar to that of the Bragg-Williams theory of positional order in alloys or the standard theory of corresponding ferromagnetic states. These omissions are not explicitly justified; other omissions, such as a treatment of the structural implications of

non-stoichiometry, or ordering of point, line or planar defects, are made explicit in the Introduction, on the grounds that they have been well covered elsewhere.

One can quibble at the margins about omissions and undefined concepts, but the final impression left is of a *tour de force*, a synthesis which has no competitor. The book deserves the close attention of adventurous inorganic and solid-state chemists, solid-state physicists (especially theoreticians) and physical metallurgists. The printing is by facsimile reproduction of typescript, but is quite clear, and in view of its size and scope the book is not overpriced.

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Pollination ecology revived

The Principles of Pollination Ecology. Third edition. By K. Faegri and L. van der Pijl. Pp. 244. (Pergamon: Oxford, 1979.) Hardback £12.50; paperback £7.50.

POLLINATION ECOLOGY is certainly not a new subject, having developed essentially during the eighteenth century and having been given new impetus by the work of Darwin in the nineteenth century. But it is a subject in which interest has lapsed somewhat until relatively recently, perhaps because its study has demanded patient and precise observation rather than more fashionable sophisticated experimentation. It may well be that this very fact is leading to a resurgence of interest in the subject, particularly on the part of the very considerable army of amateur bontanists in Britain. Already the energies of this eager body have been channelled into the detailed mapping of the British flora and now, under the auspices of the Botanical Society of the British Isles, information is being sought regarding the insect visitors to flowers.

In this academic climate, it is apt that Faegri and van der Pijl should launch a third edition of their classic survey of pollination ecology. The last edition was published in 1971 and since that time a great deal of new work has appeared in the literature. In their current bibliography about 30% of references date from this period, and this will indicate the extent of textual revision.

The book has retained its original arrangements, with chapters on general principles of abiotic and biotic

pollination, attractants, pollination and speciation, and so on; and then a series of detailed and illustrated case studies of particular plant species. The latter are particularly informative, although the drawings are sometimes so heavily stippled that structural clarity is lost. Simple line drawings would have been preferable.

In general, the text is turgid, probably because it is so heavily packed with examples and references. Although the bibliography is extensive and valuable, perhaps the book could have been improved from the point of view of undergraduate teaching if fewer examples had been dealt with in greater detail. The text generally lacks the diagrams that could have made many points more easy to grasp. Frequently an unreasonable familiarity with the literature is assumed and one is referred to the "well-known case of . . ." without further explanation. The full benefit of the text can certainly be obtained only with the backing of a good reference library.

In comparison with Proctor and Yeo's *Pollination of Flowers* (Collins: London, 1973) which, rather surprisingly, is not referred to in the bibliography, this book excels in its crystallisation of fundamental principles from a plethora of facts and observations, but falls short in its coverage of insect adaptations and in the quality of its illustrations. It is, however, a book which should stimulate further interest and research in an area of biology where plants and animals tend to interact to mutual benefit. For this reason alone it is a sadly neglected aspect of ecology which could prove a fruitful subject for practical teaching in undergraduate ecology courses. This book may lead to such developments.

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